University of Alberta

The Neuroprotective Effects of Estrogen on SK-N-SH Cells and the Cellular Mechanisms Involved

by

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Fang Ba

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T5T 2S1

Date: December 18, 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The neuroprotective effects of estrogen on SK-N-SH cells and the cellular mechanisms involved" submitted by Fang Ba in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. C. G. Benishin (Supervisor)

Dr. P. K.T. Pang (Co-supervisor)

Dr. G. B. Baker

Dr. S. T. Davidge

Dr. E. Karpinski

Dr. H. K. Yip (External Examiner)

Dr. J. J. Greer (Committee chair)

Dated: December 18 2001

Abstract

Estrogen has been shown to be neuroprotective in various studies. Estrogen replacement therapy has provided promising beneficial effects in neurodegenerative diseases, such as Alzheimer's disease. The current studies investigate the neuroprotective actions of estrogen in several models of neurotoxicity induced by different insults with the human neuroblastoma cell line, SK-N-SH cells. The insults include β-amyloid protein 25-35 (Aβ), MPTP, serum deprivation, and high density culture model. Our studies demonstrate that physiologically relevant concentrations of 17β-estradiol (E2) are neuroprotective in all cytotoxic models established. MPTP, serum deprivation, and the high density culture, but not Aβ, induce apoptosis as detected with ELISA quantification of oligonucleosomes, and DNA laddering methods. The protective effects of E2 are abolished by the addition of ICI 182,780 in the MPTP treated cells, but not in the other models, suggesting that the effect of E2 in the MPTP model is probably associated with activation of estrogen receptors.

The L-type calcium channel blocker nifedipine nearly competely blocks Aβ-induced cell death, but not MPTP. Decreased extracellular Ca^{++} concentration also restores Aβ induced cell viability loss. Western blotting analysis with anti L-type Ca^{++} channel α_1-subunit antibodies demonstrated that Aβ increases the expression of the α_1-dihydropyridine binding complex, and neuronal α_{1C} and α_{1D} subunits of L-type channels. Both E2 and nifedipine inhibit the increase in channel protein expression. MPTP induces an overexpression in α_{1C} and α_{1D}, but the increases were not modified by E2 or nifedipine. Collectively, the observed phenomena help to

illustrate the mechanisms of the neuroprotective effects of E2 in both apoptotic and necrotic cell death related to neurodegenerative changes and ischemic- or growth factor withdrawal-induced brain injury. It is proposed that E2 protection against Aβ induced cytotoxicity may occur at least partially through the regulation of expression of L-type Ca^{++} channels; the protective effects of E2 in the MPTP model is through ER-mediated anti-apoptotic pathways. Moreover, the current studies reveal that nifedipine, the acute Ca^{++} channel blocker protects neurons from Aβ toxicity through restoration of the overexpression of the channel protein. A new fundamental role of dihydropyridines will be considered in the regulation of calcium homeostasis.

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List of Abbreviations

α-KG	α-ketoglutarate dehydrogenase
4-AP	4-Aminopyridine
Aβ	β-Amyloid protein
Ach	Acetycholine
AchE	Acetylcholinesterase
AD	Alzheimer's disease
AF-1	Transactivation domain 1
AF-2	Transactivation domain 2
ALS	Amyotrophic Lateral Sclerosis
AM	Acetoxymethyl
AP-1	Activating protein-1
APOE	Apolipoprotein E
ARP	Apoptosis related proteins
βAPP	β-amyloid precursor protein
Bay K 8644	1,4-Dihydro-2,6-dimethyl-5-nitro-4-[2- trifluoromethyl phenyl]pyridine-3-carboxylic acid methyl ester
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
Ca^{++}	Calcium ion
CAD	Caspase activated DNase
$[Ca^{++}]_i$	Cytosolic free Ca^{++}
CaM	Calmodulin
cAMP	Cyclic AMP
cArb	Cytosine-arabinoside
CAT	Choline acetyltransferase
cGMP	Cyclic GMP
CgTx	ω-Conotoxin GVIA
CICR	Ca^{++} spike Ca^{++}-induced Ca^{++} release
CJD	Creutzfeldt-Jakob Disease

CNS	Central nervous sysytem
CNTF	Ciliary neurotrophic factor
CREB	Cyclic AMP response element-binding protein
CT	Computer-assisted tomography
CT 105	Recombinant carboxy-terminal 105 amino acid fragment
DAG	Diacylglycerol
DAT	Dopamine transporter
DBD	DNA-binding domain
DFF	DNA fragmentation factor
DHEA	Dehydroepiandrosterone
DHP	Dihydropyridines
DTT	Dithiothreitol.
E2	17β-estradiol
EEG	Electroencephalography
Er	Endoplasmic reticulum
ER	Estrogen receptors
ERE	Estrogen responsive element
ERK	Extracellular signal-regulated kinase
ERT	Estrogen replacement therapy
FADD	Fas-associating protein with death domain
FGF	Fibroblast growth factor
FGF-R1	Fibroblast growth factor receptor R1
GABA	Gamma-aminobutyric acid
GDNF	Glial-derived neurotrophic factor
GRP-78	Glucose-regulated protein-78
GSH	Glutathione peroxidase
HBD	Hormone binding domain
HBSS	Hank's balanced salt solution
HGF	hepatocyte growth factor
HRP	Horseradish peroxidase
HSP-70	Heat-shock protein-70

HVA channels	High voltage-activated channels
ICAD	Inhibitor of caspase-activated DNase
IEG	Immediate-early gene
IGF	Insulin- like growth factor
IKK	IkappaB kinases
IL-1β	Interleukin-1β
INF-α	Interferon-α
iNOS	Inducible NO synthase
$InsP_3Rs$	Inositol 1,4,5-trisphosphate receptors
IP_3	Inositol 1,4,5-trisphosphate
JNK	*c-jun* N-terminal kinase
K_{Ca} channels	Ca^{++} activated K^{+} channel
LTP	Long term potentiation
LVA channels	Low voltage-activated channels
M receptors	Muscarinic cholinergic receptors
MAO-A	Monoamine oxidase type A
MAO-B	Monoamine oxidase type B
MAP kinase	Mitogen-activated protein kinase
MEK	MAPK/ERK kinases
MID	Multi-infarct Dementia
Mn-SOD	Manganese superoxide dismutase
MPP+	l-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
MSA	Multiple system atrophy
mtDNA	Mitochondrial DNA
mRNA	Messenger RNA
MTP	Micro-titer plate
N receptors	Nicotinic cholinergic receptors
N-CAM	Neural cell adhesion molecule
NF-κB	Nuclear factor b kappa

NF-H	Neurofilament heavy chain
NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory agents
NT 3	Neurotrophins 3
NT4/5	Neurotrophin 4/5
$ONOO^-$	Peroxynitrite
Par-4	Prostate apoptosis response-4,
PD	Parkinson's disease
PDK	Phosphoinositide-dependent kinase
PHF	Paired helical filaments
PI- 3 K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PMCA	Ca^{++}-ATPase-type pump
PrP	Prion protein .
PS1	Presenilin 1
PS2	Presenlilin 2
PSP	Progressive supranuclear palsy
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
RAGE	Advanced glycation end products
ROS	Reactive oxygen species
sAPP	Soluble amyloid precursor protein
SDAT	Senile dementia of the Alzheimer's type
SDS	Sodium dodecyl sulfate
SERCA	Sarco-endoplasmic reticula Ca^{++}-ATPase-type pumps

SERM	Selective estrogen receptor modulator
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SOD	Superoxide dismutase
SR	Sarcoplasmic reticula
TEA	Tetraethylammonium
TNF- α	Tumor necrotic factor α
TNF-α	Tumor necrosis factor-α
TOH	Tyrosine hydroxylase
TUNEL	TdT-mediated dUTP-biotin nick end labeling
VDCC	Voltage-dependent Ca^{++} channels
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
XDH	Xanthene dehydrogenase
XO	Xanthene oxidase

Chapter 1

Introduction

1.1. Aging and neurodegeneration

1.1.1. Definition of aging

Aging is the accumulation of changes responsible for both sequential alterations that accompany advancing age and the associated progressive increases in chances of disease and death. [258] This process is common to all living things since the phenomenon of aging and death is universal. Senescent related cell death may be the major determinant of the manifestations of aging; it occurs even under optimal living conditions. Furthermore, to some extent, aging is under genetic control, since the life span differs between species and individual members of species. In addition, environmental influences are also involved.

Aging and death of single cells can be viewed as being due to the accumulation of changes produced by the environment and the intrinsic process. Similarly, aging at the multicellular level might be considered the result of inborn processes occurring in all cells, with environmental and internal influences, including the effects of the senescent cells on each other and aging changes associated with diseases.

Aging theories have long been a focus in gerontology. Based on previous research, the aging processes have been attributed to molecular crosslinking, [65-67] change in immunological function, [578, 579] damage by free radical reactions, [257, 259, 260] and to senescence genes in the DNA. [265, 266] No single theory is generally accepted to explain all phenomena.

1.1.2. Brain aging and neurodegeneration

1.1.2.1. Aging of the brain

The aging process of the brain is the subject of much scientific research because of the increase in the mean age of the population. Doubtless, aging causes changes in brain function and a decrease in physical activity. During the past decade, substantial progress has been made in the area of neurobiology of senescence. However, our knowledge of brain aging is still incomplete. Aging decreases brain function, sight, hearing, taste, memory and motor activity, thus affecting the quality of life of the elderly. Brain aging is characterized by neuronal loss that is more marked in the caudate-putamen and in some subcortical cholinergic nuclei [571] than in other areas. In normal brain aging, these changes are compensated for in terms of function and structure by the extension of dendritic ramifications of the remaining neurons. These modifications are much more obvious in degenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). [197] Experimental evidence relating to brain aging shows alteration of the cell body, nucleus, protein synthesis and RNA. [177] Aging is also characterized by deficits of various vulnerable neurotransmitters, such as cholinergic and dopaminergic systems. Alterations occur in both neurotransmitter synthesis and receptor density and/or affinity.

Several problems have been noticed in the study of aging.

1). Brain function is closely linked to other physiological functions.

2). There are no markers to distinguish normal and pathological brain aging except in few neurodegenerative disorders, such as AD.

3). The relationship between functional and chronological aging is not clear.

In particular, functional aging proceeds differently for different individuals and physiological declines may begin earlier in some individuals.

Research on neurodegenerative diseases plays a very essential part in the study of aging, and there are many theories to explain either the biological or pathological age-related and age-dependent changes.

1.1.2.2. Neurodegeneration

Diseases of the central nervous system (CNS) usually arise from damage to, or the death of, particular populations of neurons or neuroglial cells. Demyelinating diseases such as multiple sclerosis result in loss of conduction of nerve fibres following degeneration of the oligodendrocytes which form the myelin sheath around the fibres. [395] Other conditions such as AD, PD, Huntington's disease, and motor neuron disease arise from the gradual degeneration of particular populations of neurons. Similarly, the family of diseases known as spongiform encephalopathy result in the widespread degeneration of particular brain areas depending on the disease type. [427] Common features of many of these diseases are the involvement of free radicals, lipid peroxidation and the cytoplasmic or nuclear accumulation of abnormal proteins. [443, 445, 460]

1.1.2.3. Neurodegenerative diseases

The neurodegenerative diseases place an increasing medical and social burden on developed society. The diseases used to be defined and classified as separate clinical and pathologic entities. However, during this decade, detailed genetic investigations and the use of molecular pathological tools have suggested that many of these diseases share common mechanisms of pathogenesis. While these diseases have separate and distinct etiologies, they show a few common pathogenic processes that lead to cell death. [254] The etiologies and pathogeneses of these diseases remain poorly understood. However, over the past few years, the application of molecular genetics and molecular biology has allowed a clearer understanding of these diseases to begin. There are unexpected similarities and overlaps among the diseases, and it is likely that there are overlaps in the pathogenetic mechanisms that underlie the neurodegenerative processes in each of these conditions. [254]

1.1.2.3.1. Alzheimer's disease

1.1.2.3.1.1. Definition of AD

AD is a neurodegenerative disease, and this single disorder is considered to be responsible for 50 ~ 60% of all dementia cases. This disease usually occurs sporadically at age 60 or older. However, a familial form may be presented as early as age 40. The clinical presentation of AD is progressive dementia. Dementia is defined as deterioration in intellectual abilities that is of sufficient severity to interfere with social or occupational functioning [173]. The deficit involves many aspects of cognition, particularly memory and judgment. Higher cortical functions such as language, abstract reasoning, and the ability to follow directions are also commonly impaired. The actual definition of AD may vary. A variety of criteria can be applied for clinical diagnoses, therapeutic trials, epidemiological research, or pathological investigations. [128] There is no biochemical test for AD. It is diagnosed by postmortem examination of brain tissue for the presence of adequate numbers of neurofibrillary tangles and senile plaques. Magnetic resonance imaging (MRI) determination of volumetric hippocampal atrophy is considered as a sensitive indicator in early AD.[23] However, image diagnosis is not a diagnostic criterion of AD. Loss of short-term memory is the characteristic sign, but gross deterioration in all cognitive functions is seen in advanced cases.

1.1.2.3.1.2. Histopathology of AD

The histopathology of AD includes neuritic plaques, neurofibrillary tangles, loss of synapses and neurons (neuronal loss caused atrophy is shown in Fig. 1.1.), granulovacuolar degeneration, and amyloid angiopathy.[128]

Neurite (senile) plaques are swollen eosinophilic nerve cell processes occurring in spherical focal collections within the cerebral cortex, hippocampus, and amygdala. A central amyloid core surrounded by masses of neural processes undergoing various degrees of degenerative changes is characteristic. The outer margins of the plaques are usually glial cells.[127]

Neurofibrillary tangles (also called Alzheimer's Bodies) consist of large bundles of twisted tubules measuring about 20nm in diameter at their greatest width. They are derived in part from microtubules and neurofilaments. Each tubule is made up of a pair of 10nm helical filaments. The neurofibrillary tangles are found uniquely in the human brain within neurons, especially in the cerebral cortex. They have not been found in other species unless induced experimentally by injection of aluminum into the brain.[607, 620]

Granulovacuolar degeneration is distinguished by intraneuronal cytoplasmic granule-containing vacuoles occurring within the pyramidal cells of the hippocampus or in the adjacent cortex. Generalized cerebral atrophy with moderate neuronal loss is most prominent in frontal and hippocampal areas, and it is a consistent feature of AD. Sulci are widened because of narrowing of gyri. Neuronal loss is more severe in younger than in older patients and the severity is associated with an increased number of astrocytes. In addition, loss of dendrites has also been reported to occur with aging, especially in demented individuals.

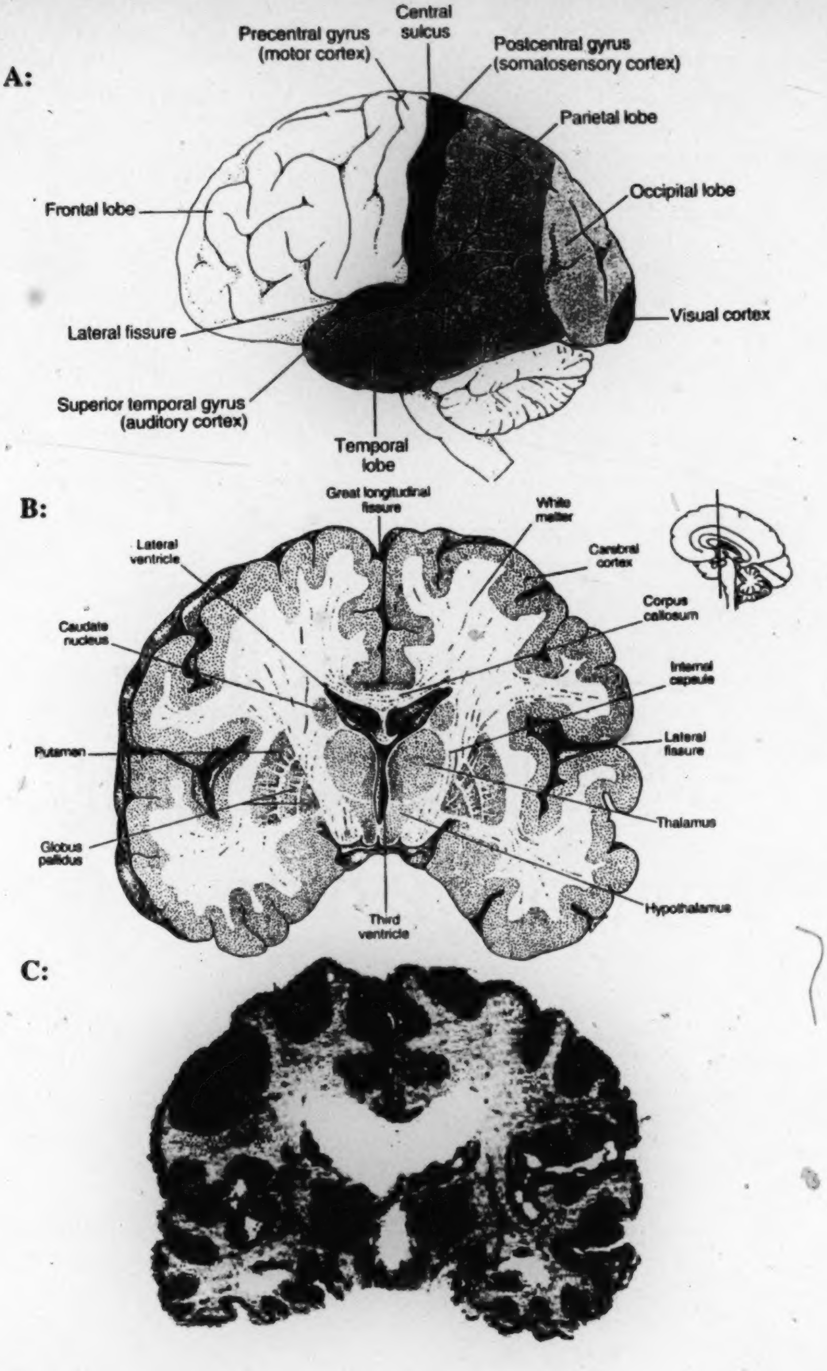

A:
Central sulcus
Precentral gyrus (motor cortex)
Postcentral gyrus (somatosensory cortex)
Parietal lobe
Frontal lobe
Occipital lobe
Visual cortex
Lateral fissure
Superior temporal gyrus (auditory cortex)
Temporal lobe
B:
Great longitudinal fissure
White matter
Lateral ventricle
Cerebral cortex
Corpus callosum
Caudate nucleus
Internal capsule
Putamen
Lateral fissure
Thalamus
Globus pallidus
Third ventricle
Hypothalamus
C:

Fig. 1.1. Pathological brain atrophy of AD patients.

Normal human brain structure of the lateral aspects (A) and a coronal section through the cerebral hemisphere (B). AD patient brain shows considerable atrophy and dialation of the lateral ventricles (C).

1.1.2.3.1.3. Etiology of AD

The cause of AD is still unknown. However, in the past 20 years, there has been abundant research demonstrating risk factors and possible causes of the disease. Research has addressed the following aspects:

1). What initiates the process of cell dysfunction, when does it start, and what are the steps in the cascade of events leading to cell death?

2). What factors determine the specificity of cell dysfunction and death?

3). What are the critical interactions between the various cellular components and the cascade of the events?

The major theories of the etiology of AD are summarized below.

Neurotransmitter disturbances in AD

Acetycholine (ACh) deficit contributes to memory loss and cognitive dysfunction. ACh is synthesized by choline acetyltransferase (CAT). The major source of CAT is in the basal forebrain. This region is affected early in the course of AD, leading to a marked and consistent deficit in CAT and ACh synthesis. Previous results have shown that CAT levels are reduced from 58% to 90%. [251] These reductions correlate with the severity of the dementia syndrome. [599] Acetylcholinesterase (AchE), the enzyme responsible for the degradation of ACh, is also reduced in AD. Muscarinic (M) and nicotinic (N) cholinergic receptors have been identified in the brain. M1 receptors are relatively preserved in AD, whereas the M2 and nicotinic receptors are largely decreased. [621] Although the M1 receptors are preserved in number, they may not be fully functional.

Non-cholinergic transmitters and neuromodulators are also affected in AD. Serotonin is reduced by 50 to 70%, GABA by 50%, somatostatin by 40 to 60%, and nonepinephrine by 30 to 70%. [454] Receptors for serotonin, glutamate, and somatostatin are also decreased in AD. [595]

Genetic factors in the genesis of AD

Epidemiological surveys have suggested that a positive family history, prior exposure to significant head injury, lack of early childhood education, and possibly certain other factors may contribute to the occurrence of AD. However, the positive family history is the most important risk factor. These genetic risk factors are probably inherited as one or more single gene traits that contribute to the etiology of AD. Some studies also suggest that familial factors may account for approximately 40 to 60% of the risk for AD. [311]

To date, molecular genetic studies have identified four different genes associated with increased risk for AD. Mutations in the β-amyloid (Aβ) precursor protein (βAPP),[115] presenilin 1 (PS1), [520] and presenilin 2 (PS2), [520] are generally associated with early-onset AD. These three genes relate to greater than 85% of the occurrence of the disease, and have a clear-cut autosomal dominant pattern of inheritance. Generally, the clinical and pathological phenotypes of patients affected with AD as a consequence of these mutations are indistinguishable from those of "sporadic" AD. Thus, some cases with βAPP or PS1 mutations might have occasional cortical Lewy bodies (i.e. eosinophilic structures located within the cytoplasm of neurons. They are thought to be the result of altered neurofilament metabolism and/or transport due to neuronal damage and subsequent degeneration, causing an accumulation of altered cytoskeletal elements). There is no uniform or clinical or pathological feature which *a priori* distinguishes these cases. However, mutations in all three of these genes are associated with increased production of $A\beta_{42}$ peptides and/or total Aβ peptides, [29, 114] which are causative features of AD. In contrast, the majority of cases of late-onset AD do not show overproduction of $A\beta_{42}$.

In contrast to the rare, early-onset forms of autosomal dominant AD associated with PS1, PS2 and βAPP mutations, the ε4 allele of apolipoprotein E (APOE) is associated with a dose-dependent increase in risk and earlier age-of-onset for the familial form of AD. However, APOE contributes to the occurrence of sporadic AD with an earlier onset as well. The sporadic form typically presents later in life (more than 55 years). [497] There is some evidence that APOE may be a comparatively weaker risk factor in the "oldest old" (over the age of 85 years). [478] The mechanism by which the ε4 allele of APOE contributes to increased susceptibility for AD is unclear. Several different theories have hypothesized that APOE might involve effects on non-specific regenerative and repair mechanisms, on the *tau* microtubule-associated protein, and on extracellular βAPP and Aβ processing. [490]

It is known that the currently identified AD susceptibility genes account for only approximately half of the genetic factors causing AD. Additional AD genes have also been investigated. Hendriks (1996) and Lendon (1997) have revealed that gene mutations involving chromosomes 21, 14, and 1 cause AD. [270, 345] The mutations are inherited in an autosomal dominant mode of transmission and, when present, usually cause familial early-onset AD. Among all the mutations, Down syndrome was the first genetic syndrome to be linked to AD. Down syndrome patients over age 30 almost universally have AD-type pathology in the brain, and the prevalence of dementia in Down syndrome also increases with age. [128] Patients with Down syndrome have three copies of chromosome 21, which bears the gene for the βAPP, and have increased production of Aβ, the toxic fragment derived from βAPP. [253] In addition, chromosome 14 mutations closely involve PS1 mutations, and chromosome 1 mutations closely involve PS2 mutations. The effect common to all mutations is the overproduction of Aβ. Recent studies have identified a putative locus on chromosome 12 in a subset of families. Other unconfirmed loci have been mapped on chromosomes 1, 9, 10, and 19. [536] Thus, a long list of candidate genes is under investigation to further demonstrate the genetic causes of the disease.

Cerebral accumulation and cytotoxicity of Aβ

It is proposed that progressive accumulation of Aβ in the brain initiates a complex multicellular cascade that includes microgliosis, astrocytosis, neuritic dystrophy, neuronal dysfunction and loss, and hence synaptic insufficiency that results in neurotransmitter alterations and impaired cognitive functions. [517] Aβ is a peptide fragment, principally 40 or 42 amino acids long, that is proteolytically cleaved from a large precursor polypeptide, βAPP. Once generated, Aβ can apparently exist in a number of forms, including monomers, dimmers, higher oligomers and polymers, the last of these including those that constitute the amyloid neurofibrillary tangles.

Aβ is a normal product of βAPP processing. The major route of βAPP processing is by α-secretase, an enzyme that cleaves within the βAPP sequence. Cleavage by β- and α-secretases at the N- and C-terminal ends of the βAPP sequence liberates the Aβ polypeptide, which can subsequently be secreted from cells. The major form of Aβ that is secreted contains 40 amino acids ($A\beta_{40}$). However, minor species containing 42 or 43 amino acid residues ($A\beta_{42/43}$) are also produced. These extended forms of Aβ aggregate more readily and may seed amyloid fibril polymerization during the early stages of plaque formation. [278]

Studies on the genetics of AD and on βAPP transgenic mice provide compelling evidence that a disturbance in βAPP metabolism or function plays a predominant role in AD pathology. However, these studies do not prove that Aβ is the causative agent. Promising evidence that implicates Aβ in the pathogenesis of AD comes from the observation that Aβ peptides are toxic to neurons in culture. [532, 618] This toxicity is enhanced when the peptides are incubated (from hours to days) to age. The incubation increases amyloid fibril formation and aggregation. Although the process of aging *in vitro* increases the number of amyloid fibrils formed from Aβ, this is not proof *per se* that fibrils are the major toxic form of Aβ. It is likely that the levels of soluble oligomeric species of Aβ are also increased by the process of aging.

The mechanism of Aβ neurotoxicity is unclear. Some studies suggest that Aβ can disrupt calcium homeostasis, [376] perhaps by forming a pore structure similar to L-type voltage-dependent calcium channels (VDCCs) [364, 569] on the cell membrane. Aβ may reduce Na^+/K^+-ATPase activity, [364] thereby influencing membrane depolarization. Other studies suggest a role for reactive oxygen species (ROS) in Aβ toxicity. [35, 37] Disturbances in redox $^{\text{potential}}$ may lead to disruption of calcium homeostasis, as ROS can impair ATPase activities. Aβ may cause lipid peroxidation and affect superoxide dismutase (SOD), which may contribute to its neurotoxicity in culture.

A membrane receptor of Aβ is considered to transduce the effects of Aβ. [150] Moreover, the receptor for advanced glycation end products (RAGE) [615] has been implicated in Aβ toxicity. Studies by Yang *et al.* [617] suggest that Aβ may also bind an intracellular hydrosteroid dehydrogenase known as ERAB. The neurotoxicity may be mediated by an indirect action of Aβ on a nonneuronal cell. For example, microglial cells are often found in association with neuritic plaques, and Aβ has been shown to activate microglia in culture, [522] increasing ROS and cytokine production. [118, 545] Therefore, the possibility that Aβ stimulates release of an unidentified neurotoxic agent from a nonneuronal compartment must also be considered. Recent work by Geula *et al.* [206] has shown that when aged aggregated Aβ is injected into the brains of old rhesus monkeys, it is neurotoxic. However, injection of the same material into young monkeys has little toxic effect. This suggests that although Aβ may be pathogenic, there must be other age-related susceptibility factors that are also important to generate a toxic reaction *in vivo*.

The toxicity exerted by Aβ in AD is demonstrated in the following flowchart:

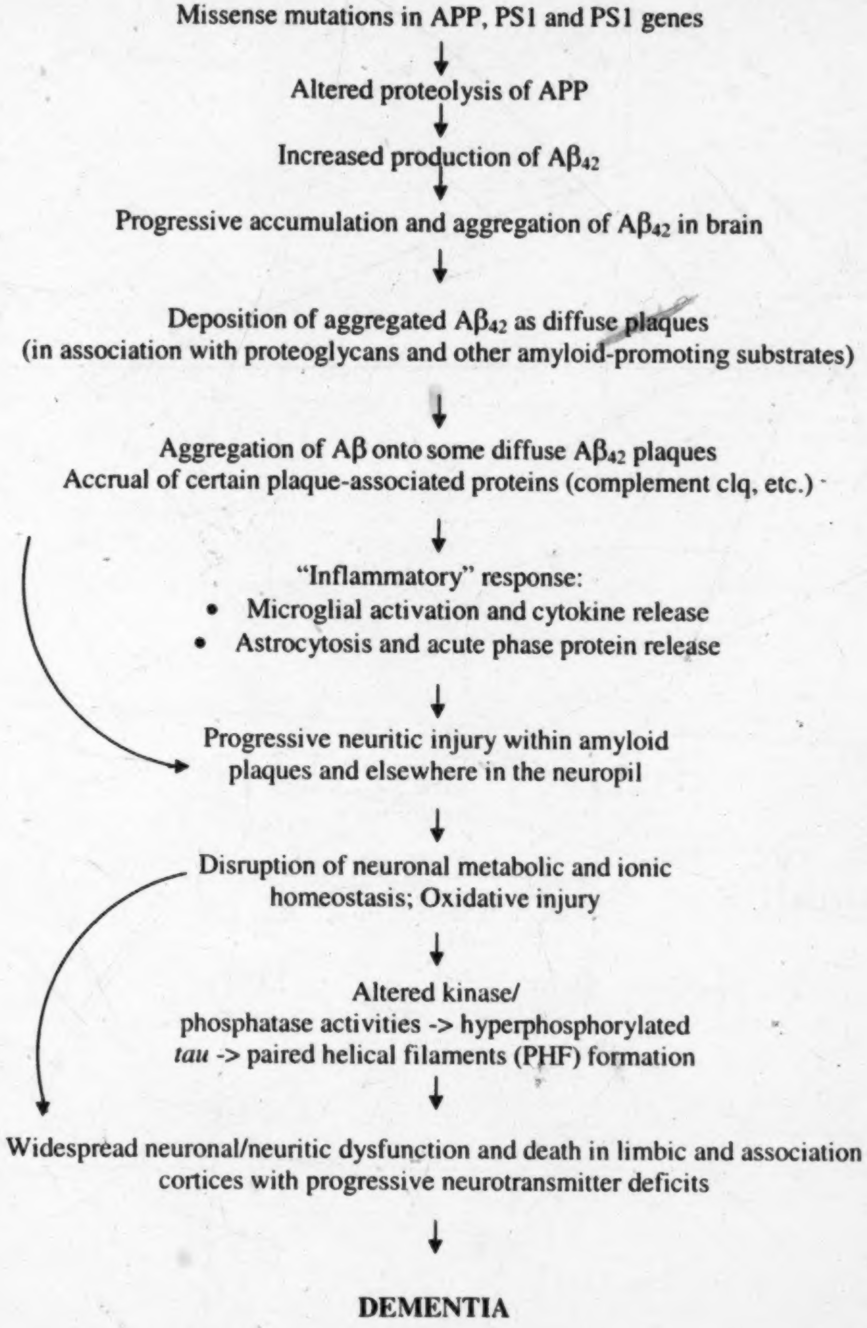

Missense mutations in APP, PS1 and PS1 genes
Altered proteolysis of APP
Increased production of $A\beta_{42}$
Progressive accumulation and aggregation of $A\beta_{42}$ in brain
Deposition of aggregated $A\beta_{42}$ as diffuse plaques
(in association with proteoglycans and other amyloid-promoting substrates)
Aggregation of $A\beta$ onto some diffuse $A\beta_{42}$ plaques
Accrual of certain plaque-associated proteins (complement clq, etc.)
"Inflammatory" response:
• Microglial activation and cytokine release
• Astrocytosis and acute phase protein release
Progressive neuritic injury within amyloid
plaques and elsewhere in the neuropil
Disruption of neuronal metabolic and ionic
homeostasis; Oxidative injury
Altered kinase/
phosphatase activities -> hyperphosphorylated
tau -> paired helical filaments (PHF) formation
Widespread neuronal/neuritic dysfunction and death in limbic and association
cortices with progressive neurotransmitter deficits
DEMENTIA

Fig. 1.2. Hypothetical sequence of the pathogenic steps of Aβ metabolism in AD. [517]

The brain microenvironment and early function loss, and the conversion to AD

Progressive cell loss in specific neuronal populations by apoptotic cell death is the pathological hallmark of many neurodegenerative diseases. In AD and other age-related neurodegenerative diseases, the brain appears to accumulate a set of conditions that may increase cellular vulnerability to apoptosis. Such pro-apoptotic conditions include environmental and/or genetic risk factors, including Aβ, reduced energy metabolism, oxidative damage, reduced glutamate transport, mitochondrial damage, genomic and mitochondrial DNA (mtDNA) damage, and PS mutations. [121, 122] The accumulation of risk factors with age and the accelerated accumulation of these risk factors in AD suggests that apoptotic cell death is a mechanism that contributes to the progression of dementia. Therefore, definition of the mechanisms driving apoptosis is fundamental, revealing the pathogenesis of the disease and developing therapeutic strategies to delay disease progression.

Cotman and Anderson suggest that pro-apoptotic conditions occur in local neurophil environments and expose the processes of neurons to such influences. [121] Thus, the processes of neurons may be prime targets for early degeneration, resulting in the loss of synaptic connectivity. In fact, recent data support the hypothesis that neuronal processes are susceptible to local degeneration, and the degeneration process shows certain similar mechanisms to apoptosis cascades. Furthermore, conditions affecting the entire neuron may exert their initial impact on the processes of these cells, which may be more sensitive to insult than the cell body. In these cases the local environment (e.g., chronically reactive, dysfunctional, or degenerating glial cells) could further exacerbate and accelerate the loss of synaptic connectivity.

Neuronal processes have the machinery to activate local degenerative events and cleave key substrates necessary for their function, such as cytoskeletal proteins and other proteins involved in synaptic transmission. The activation of such local sets of events would not preclude a later involvement of the entire cell. Some hypotheses suggest that neurites may undergo an apoptosis-like mechanism of degeneration. The degeneration or dysfunction of microglia would potentially increase the vulnerability of neuronal processes to insult and thus facilitate the breakdown of synaptic connectivity.

Inflammation and the AD brain

There are abundant studies suggesting that inflammation may play an important role in the development and progression of AD. Inflammation is not the primary cause or etiology of AD. Rather, inflammation is typically a secondary reaction to some primary pathogens or processes. Inflammation is one of the mechanisms that damage the brain in AD. The pathophysiological relevance of inflammation to AD neurodegeneration has received increased attention for several reasons. First, many of the inflammatory mechanisms that have been uncovered in the AD brain [7] are overtly cytotoxic in the periphery. [330] Since the brain is an organ exquisitely sensitive to inflammation; inflammatory responses would therefore also be cytotoxic in the brain. Second, up-regulated inflammatory mechanisms are colocalized in the AD brain within those regions that exhibit high levels of AD pathology, and are absent or minimal in brain regions with low AD pathologic susceptibility. [7] Third, at the microcellualr level, inflammatory mediators are most highly expressed in the vicinity of Aβ deposits and neurofibrillary tangles, the classical histopathological hallmarks where neurodegenerative changes occur. [517] Fourth, patients without a history of dementia but who nonetheless exhibit sufficient limbic Aβ and tangles at autopsy show only modest elevations of inflammatory markers, slightly greater than those in typical nondemented elderly patients but dramatically less than those of AD patients. Fifth, direct evidence of inflammatory toxicity can be observed in the AD brain. Sixth, many clinical studies have suggested that conventional anti-inflammatory drugs may delay the onset or slow the progression of AD. Additionally, numerous basic science studies have shown that the pathologic entities in AD, i.e. Aβ aggregates and neurofibrillary tangles, stimulate multiple mechanisms of inflammation. [589] As in the periphery, AD inflammation is made up of a complex set of interactive, feedforward and feedback mechanisms. Through complement receptors expressed by microglia, for example, complement activation fragments can induce cytokine production. Cytokines, in turn, can induce acute-phase proteins, chemokines, free radicals, additional cytokines, and more complement [486]. In the end, the multiple interactions among the many inflammatory mediators that have been detected in the AD brain make it difficult to assert that any one mediator or set of mediators is more important or more primary than any other, and whether inflammation is causative of or secondary to AD.

Oxidative stress in AD

Free radical formation has been another focus in the etiology research on AD during the past decade. It is hypothesized that senile dementia of the Alzheimer's type (SDAT) may be the result of mtDNA mutations, early in development and after germ cell segregation, that impair oxidative phosphorylation and increase production of O_2^{-} and H_2O_2. Replicative segregation distributes the mutated mtDNA to the cells of the developing organism. With advancing age, cellular dysfunction occurs first in areas containing the mutated mtDNA of the brain associated with AD. Cell damage and death are attributed to random free radical damage secondary to falling ATP production and increasing formation of $O_2^{\cdot-}$ and H_2O_2 from aging and defective mitochondria. The increasing oxidative stress contributes to cell damage and eventual death in part by impairing cellular control of cellular calcium ion (Ca^{++}) concentration. A sustained increase in Ca^{++} disrupts the cytoskeleton and activates Ca^{++} - dependent catabolic enzymes.

The free radical theory of aging states that the aging process is the result of progressive and cumulative insult to the macromolecules of the cell producing oxidative modifications and loss of function. [259, 260] The oxidative damage parallels the distribution of amyloid plaques, neurofibrillary tangles, and reactive microglia. In fact, of the factors that have been associated with AD, all of them either directly or indirectly give rise to oxidative stress. [96] In addition, senescent mitochondria are less efficient in generating ATP and leak more free radicals, and are also less effective in sequestering Ca^{++} (50% less). [443] This may be important for the disruption of normal synaptic architecture and the loss of synaptic connections in AD. Since the major flux of Ca^{++} occurs in the processes of the neuron (dendritic and axonal), differences in mitochondrial function in these areas could increase vulnerability to damage. [96]

Mitochondrial dysfunction may be linked to the other neuropathological hallmarks of AD, including senile plaques and neurofibrillary tangles. Studies have shown that metabolic defects can alter βAPP processing. *In vitro* energy impairment can lead to a reduction in βAPP and increased production of either Aβ or Aβ containing C-terminal fragments. [340] Furthermore, oxidative damage has been shown to increase intracellular $A\beta_{42}$. [511] Oxidative damage may also lead to crosslinking and impaired solubility of Aβ. [52] Oxidative injury has also been shown to lead to intramolecular crosslinks, which can contribute to the generation of paired helical filaments. Oxidation of critical cysteine residues seems to be associated with aggregation of *tau* proteins in the paired helical filaments. Reduced ATP generation can also lead to increased activation of several kinases that phosphorylate *tau* proteins. [33, 597] Most importantly, either necrosis or apoptosis occur at the end of the cascade of oxidative stress, and these are the major pathological changes in the disease.

Impaired cerebromicrovascular perfusion

Studies have shown that multiple pathologic events in AD are triggered by impaired cerebral perfusion originating in the microvasculature and are affecting the optimal delivery of glucose and oxygen. This results in a breakdown of energy metabolism in brain cell biosynthetic and synaptic pathways. [135-137] De la Torre [136] proposed that two factors must be present before cognitive dysfunction and neurodegeneration can be expressed in the AD brain: (1) advanced aging and (2) presence of a condition that lowers cerebral perfusion, such as seen with vascular risk factors. [137] Aging introduces a normal but potentially menacing process that lowers cerebral blood flow in proportion to increased aging, while an impaired perfusion places vulnerable neurons in a state of metabolic compromise, leading to a death pathway.

The impaired cerebromicrovascular perfusion is probably not the cause of AD, but such a condition may accelerate the progress of the disease.

Other AD theories

In AD research, profound progress has been made during the past decade in understanding the cellular aspects of etiology leading towards a comprehensive theory of the causes of AD and its diagnosis. One of the unresolved challenges is to determine the precise cascade of events leading to the proximal causes of this degenerative process. There are many other theories. Carney and co-workers [96] have established a theory that focuses on the relationship between oxidative stresses and the decrease in proteases seen in aging. Proteases act to govern the acceleration of the pathology of aging. As damage from oxidative stress increases, the loss of proteases in an "at-risk" person leads to symptoms of AD. They suggested that controlling the damage of proteases may help to control the onset or progression of AD. Nixon *et al.* [438] have shown that cathepsins and calpains acting directly or indirectly through other proteolytic pathways and cellular signaling cascades may promote β-amyloidogenesis, neurofibrillary pathology, as well as mediate neurodegeneration in AD.

A new hypothesis of the causation of AD states that AD pathogenesis results from disruption of cholesterol uptake and metabolism and that this in turn results in abnormal trafficking of membrane proteins critical to normal neuronal function and synaptic plasticity. [358] A plasticity-based theory of the pathogenesis of AD has been put forward by Mesulam's group. [401] They indicate that the resultant intensification of the plasticity burden leads to an initially adaptive up-regulation of *tau* phosphorylation and βAPP turnover, and to the subsequent formation of neurofibrillary tangles as independent consequences of excessive plasticity-related cellular activity. Eventual loss of neurons, dendrites, and the synapses is the ultimate expression of plasticity failure. The "fatal attractions" hypothesis of Lee [564] describes a plausible unifying mechanism that accounts for the onset/progress of AD and a large number of other seemingly unrelated neurodegenerative disorders characterized neuropathologically by filamentous brain lesions formed by different proteins. This hypothesis predicts that the abnormal interaction between normal brain proteins alters their conformation and promotes the assembly of these pathological structures into filaments that progressively accumulate as intracellular and extracellular fibrous deposits in the CNS. Further, the transformation of the normal proteins into pathological conformers is predicted to result in losses of critical functions. The disease proteins or their progressive accumulation into filamentous aggregates are predicted to acquire neurotoxic properties, leading to dysfunction and death of affected brain cells.

Aluminum has also been considered as a cause of AD. Aluminum is accumulated in human neurons harboring neurofibrillary tangles. Patients who have undergone repeated kidney dialysis with aluminum-rich dialysis solutions have elevated brain aluminum levels. Moreover, some brain enzymes can be inhibited by aluminum, and aluminum salts can interfere with the transport of certain proteins down the axons of neurons. However, as our understanding of the pathogenic mechanisms of AD has advanced, more recent studies have established a new theory that exposure to aluminum is not a risk factor for the development of AD. [64, 421, 422]

1.1.2.3.1.4. Risk factors for AD

As discussed above, vulnerability genes are involved in the occurrence of AD. Other risk factors for AD are family history, age, female gender, history of head trauma, head circumference and brain size, and lower intelligence. [128]

A family history of AD increases risk for developing the disease. The frequency of AD in first degree relatives of a AD patient is two- to four-fold the risk for elderly individuals without a family history. [81, 408] Age is a powerful risk factor. Both the prevalence and the incidence of AD double approximately every 5 years after the age of 60. Prevalence rises from approximately 1% in 60- to 64-years olds to about 35 to 40% in those over the age of 85. [128] The incidence of AD increases dramatically in women after menopause. More women live into the higher risk period of AD, and therefore women are more common in the population of AD patients. Synaptic relationships between neurons may be disturbed by diffuse axonal injury sustained at head injury. The predisposing injury factors increase the likelihood of the later appearance of AD. [391] In addition, smaller brain size has also been associated with an earlier onset of AD, probably due to fewer nerve cells with fewer synaptic connections, which may confer poorer protection against symptoms of AD. [415] Lower cognitive function has been associated with a higher risk of AD. Higher intelligence is associated with larger brain size and more rapid nerve conductance. [138] These factors may increase cognitive reserve and thus may prevent the onset of AD, whereas lower intelligence increases the likelihood of AD manifestation.

1.1.2.3.1.5. Treatment of AD

Several therapeutic strategies exist in current AD treatment. Currently available AD-specific therapies include symptomatic approaches based on enhancement of cholinergic function, neuroprotective approaches utilizing antioxidant agents, compensation for the immune and inflammatory responses, etc. Neuroprotective agents have been successfully employed to slow the progress of AD. As noted above, inflammatory pathways are activated in AD, and non-steroid anti-inflammatory agents (NSAIDs) may slow the progress and defer the onset of AD. Steroids or NSAIDs with acceptable side-effect profiles for chronic use in the elderly might represent useful approaches in the treatment of AD. [140, 537]

Reduced Aβ production is an obvious therapeutic target in AD. In diseases such as Down syndrome, in which βAPP is overexpressed, regulation of the βAPP gene could be targeted by genetic therapies that would modulate gene activity. Increasing or amplifying the α-secretase pathway in favor of the β- and γ-secretase pathways might reduce the production of Aβ. Blockade of β- or γ-secretase offers another means of decreasing Aβ production. Finally, Aβ aggregation might be blocked through effects on APOE-4 or through agents that block the conformational change of Aβ. [350] Genetic therapy is a newly developed strategy, and has a promising future in AD treatment.

1.1.2.3.2. Parkinson's Disease

1.1.2.3.2.1. Definition of PD

PD, or idiopathic Parkinsonism, is the second most common age-related neurodegenerative disorder in human, affecting more than 1 million people in North America. [470] The incidence of the disorder is estimated between 1:100 ~ 1:500. PD is a movement disorder with the peak mean onset in the sixth decade of life, and it is characterized by resting tremor, rigidity, bradykinesia or slowness, gait disturbance, and postural instability. [445] Pathological features of PD include degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) coupled with intracytoplasmic inclusions known as Lewy bodies. Patients with PD show beneficial response to dopamine replacement therapy. It has been observed that the administration of the dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can cause Parkinsonism.

1.1.2.3.2.2. Epidemiology and symptoms of PD

PD has been referred to as an ailment of old age. However, recent studies indicate that the incidence of new cases reaches a peak at about age 55. Thus, about half the victims are affected during their most productive years. About 10% of cases appear before age 40. It strikes both men and women, with a slightly higher percentage of men than women affected. Some cases are steadily progressive, some progress in spurts, and some remain mild for years. There are many people who have managed to live active lives for more than 25 years after the appearance of the first identifiable symptoms. Parkinsonism is rarely fatal, but in advanced, uncontrolled stages its disabling effects can make a patient more likely to succumb to other ailments. [547]

The major visible symptoms of PD include tremor, rigidity and bradykinesia. Other major symptoms that develop over a period of time, if untreated, include stooped posture, a shuffling gait, "frozen" or masklike facial expression, and a tendency to speak softly and indistinctly. Involuntary movement affected by Parkinsonism includes walking, swallowing, digestion, elimination, etc.. Parkinsonism tends to slow down or hamper most of these functions.

Recent research on the brain has revealed that movement, emotions, and thought processes are closely interrelated. Parkinsonism has effects on emotions and thought as well as on physical movement, and *vice versa*. Moreover, there appear to be three types of depression that may affect a Parkinsonian patient. They are reactive depression, a response to fears and caused by the ailment; endogenous depression, a symptom of Parkinsonism that may have chemical or biological causes; and depression that is common to old age, and is only coincidental with Parkinsonism. [626]

It is often difficult to diagnose the early stages of PD because the patient's symptoms may be vague. Often, diagnosis will be made only after the patient's tremors become readily apparent and one or more of the other classic symptoms appears. There is no specific test for the presence of the disease; rather, diagnosis is made on a thorough neurological examination. Computer-assisted tomography (CT) scans and MRIs may be ordered to rule out other diseases. There are multiple forms of PD, but the symptoms associated with all forms of the disease are similar. It is important to diagnose the specific form and the progression of PD because the treatment program will vary accordingly. [75]

1.1.2.3.2.3. The neuropathological basis of PD

Early researchers localized the abnormalities to a few sites in the upper brain stem, especially in the substantia nigra (SN) of the mesencephalon. In the mid 1950s, James Greenfield concluded that the real pathologic location of PD was not confined to these sites, but also occurred at a few other sites where pigmented neurons cluster (e.g., the locus ceruleus of the pons and the dorsal vagal nuclei of the medulla). Shortly afterward, the pigmented cells in the SN were found to synthesize dopamine. [287] More recent observations have added other dopamine-producing regions to the list of affected neuronal populations, specifically the ventral tegmental region of the midbrain (near the SN). This locus of cells projects its axons to the basal forebrain and frontal cerebral cortex and is thought to contribute to normal cognitive and behavioral function. Mild-to-moderate depletion of dopamine in the ventral tegmental-basal forebrain-cortical circuit occurs consistently in PD. It may contribute to the cognitive-behavioral problems, especially the slowed mental processing experienced by many nondemented patients with PD. Autonomic neurons in the hypothalamus, thoracic spinal cord, and even the myenteric plexus of the esophagus and colon have also been shown to degenerate, but the severity of this degeneration varies. [365]

For reasons as yet unknown, the pigmented, melanin-containing, dopamine-producing neurons of the SN undergo selective and progressive cell death during the course of the illness at rates that roughly parallel the clinical course of the patient's progressive disability. Microscopic analysis of the brain in patients with PD shows severe loss of neurons and proliferation of reactive glial cells in the anteriormost layer of neurons of the SN and severe secondary depletion of dopamine stores in the caudate and putamen. It is estimated that depletion of total stores of dopamine in the basal ganglia must exceed 50% to 75% in this preclinical or subclinical phase of the degenerative process before symptoms appear.

Another unsolved mystery is the true significance of the Lewy body typically found in the cytoplasm of the nigral neurons that survive to be visualized at postmortem examination. The Lewy body is also found abundantly in neurons of the cerebral cortex in approximately 30% of AD patients who have a mixture of AD and widespread cortical Lewy bodies. Dementia can be an early feature of one of the less common atypical Parkinson syndromes, known as diffuse Lewy body disease, a combination of relatively mild Parkinsonism and early-onset, progressively severe dementia.

Speculation about the cause of the selective nigral cell death that typifies PD has been broad, ranging from intoxication by an environmental toxin to a genetically mediated vulnerability of melanized neurons to the killing effect of uncontrolled oxygen free radicals.

1.1.2.3.2.4. Etiology of PD

It has been postulated that PD is initiated by the interaction of genetic predisposition and environmental factors. [305] Such interactions induce mitochondrial respiration failure, [406] and oxidative stress within nigral neurons and lead to cell death. [199] Recent information firmly indicates that genetic factors at least play a role in the etiology of PD. This role, however, probably varies in degree from one individual to another. Evidence for a variety of other etiological factors has been amassed from epidemiological studies, animal models and molecular and cellular biology. Genetic factors, infectious and immunological abnormalities, the effects of aging, toxins (endogenous and exogenous) and other environmental factors may all contribute to the development of PD. Loss of nigral dopaminergic neurons may be mediated by combinations of oxidative free radical toxicity, impaired mitochondrial function, "weak excitotoxicity" and abnormal handling of cytoskeletal proteins, all of which may shift the balance of apoptotic cell death. [538]

Environmental Factors

The specific etiology of PD is not known. Epidemiological studies indicate that a number of environmental factors may increase the risk of developing PD. These include exposure to well water, pesticides, herbicides, industrial chemicals, wood pulp mills, farming, and living in a rural environment. [445, 549] A number of exogenous toxins have been associated with the development of Parkinsonism, including trace metals, cyanide, lacquer thinner, organic solvents, carbon monoxide, and carbon disulfide. There have also been indications for the possible role of endogenous toxins such as tetrahydroisoquinolines and β-carbolines. However, no specific toxin has been found in the brain of PD patients, and in many instances the Parkinsonism seen in association with toxins does not show typical Lewy body as seen in PD. The most compelling evidence for an environmental factor in PD relates to the toxin MPTP, byproduct in the illicit manufacture of a synthetic meperidine derivative. Drug addicts who received MPTP developed a syndrome that strikingly resembled PD, both clinically and pathologically. [339] MPTP induces toxicity through its conversion in astrocytes to the pyridinium ion, 1-methyl-4-phenylpyridinium (MPP^+) in a reaction catalyzed by monoamine oxidase type B (MAO-B). [528] MPP^+ is then taken up by dopamine neurons and causes a mitochondrial complex I defect similar to that found in PD. [434] It has been suggested that MPP^+ enhances glutamate toxicity to DA neurons in culture, and MPTP/MPP^+ toxicity can be prevented by non-competitive NMDA antagonists. [293] The acute phase of MPTP/MPP^+ toxicity might be associated with excessive depolarization-induced stimulation of glutamate receptors. Neurons are then more susceptible to the influx of Ca^{++} because of impaired mitochondrial function. [31] These observations support the possibility that an environmental factor might cause PD; however, no MPTP-like factor has been identified in PD patients to date.

Genetic Factors

There has been considerable interest in the potential role of genetic factors in the etiology of PD. Research from the past decade reveals that genetic predisposition appears to play an important role in nigral cell death in PD, but direct proof is lacking. Approximately 5–10% of PD patients have a familial form of Parkinsonism with an autosomal-dominant pattern of inheritance. [211] Large pedigrees have been identified where members in different generations suffer from PD. In addition, the incidence of PD is greater in family members with a history of PD than in age-matched controls. [608] One study revealed no difference in concordance between monozygotic and dizygotic twins of PD patients aged 60 years or older. However, a significantly increased incidence was observed in monozygotic twins who developed PD at less than 50 years of age. [548] This suggests that genetic factors are important in young-onset patients but are not likely to play a major role in patients with sporadic PD. A number of candidate genes have been screened and found not to be associated with an increased risk of PD. These include genes for APOE-4, tyrosine hydroxylase (TOH), glutathione peroxidase, catalase, SOD-1 and 2, and the dopamine D2, D3, and D4 receptors. [200] Some, but not all studies, have detected polymorphisms in the genes encoding cytochrome P4502D6 (CYP2D6) and MAO-A and –B. However, these gene markers account for only a small number of PD cases at most, as the allelic frequencies are relatively uncommon. More recently, an association has been demonstrated between PD and the presence of a slow acetylator phenotype. This association was stronger in patients with familial rather than sporadic PD, suggesting the possibility of genetic abnormality in PD etiology. [445]

There has been an extensive search for a mutation in the mitochondrial genome, based on the finding of a defect in mitochondrial complex I in the SNc of PD patients. Complex I is composed of 41 subunits, 7 of which are encoded by mtDNA. MtDNA is a circular double-stranded molecule that is much more likely to undergo mutation than is nuclear DNA. In one study, a 5-kb deletion was detected in the mitochondrial genome, but it was identical to that found in a normal aging population. [407] The same group sequenced total mtDNA in five sporadic PD patients and noted different point mutations in a subunit of complex I in each. However, no disease-specific mutation was identified, and the mutations found did not associate with a familial form of PD. MtDNA mutations can be transmitted maternally, but most studies have failed to detect a maternal pattern of inheritance in PD. The mtDNA mutations seem to involve sporatic PD only, as the majority of patients with a mitochondrial mutation (e.g., deletions and the A3243G mutation) do not have a positive family history.

More recent research has indicated that PD is linked to the q21–23 region of chromosome 4 in a large Italian-American family known as the Contursi kindred. [469] Patients had a relatively early age of onset but otherwise demonstrated typical clinical and pathological features of PD, including Lewy bodies. Subsequently, a mutation was detected in the gene that encodes for the protein α-synuclein in this family. This was also found in several apparently unrelated Greek families. Sequence analysis demonstrated that the mutation consisted of a single base pair change from G to A at position 209 (G209A), resulting in an alanine to threonine substitution at position 53 (Ala53Thr) in the α-synuclein protein. In the affected families, 85% of patients who expressed the mutant gene had clinical features of PD, whereas this mutation was not seen in any of 314 controls. A second mutation in the α-synuclein protein (Ala30Pro) has recently been described in a German family. These findings provide strong evidence that a single mutation in the human α-synuclein gene is sufficient to account for the PD phenotype. [327]

α-Synuclein is a small protein of 140 amino acids. Mutations in the α-synuclein gene have not been identified in patients with sporadic PD. Immunocytochemistry has demonstrated that α-synuclein is an abundant component of Lewy bodies even in patients with familial or sporadic PD who do not have the gene mutation. This suggests that accumulation of α-synuclein may be central to the development of PD. Recent studies note that apoptosis of nigral neurons is associated with up-regulated expression of the α-synuclein gene and protein. [91] In this model, α-synuclein staining is widespread in the SN but is confined to surviving neurons rather than those undergoing apoptosis. These findings may indicate that up-regulation of α-synuclein may be related to anti-apoptosis or to the promotion of neuronal survival under conditions of stress.

The association of a PD syndrome with both MPTP and mutations in α-synuclein suggests that either an environmental or a genetic factor can cause PD. However, it is unlikely that in the majority of cases PD will be explained by a single cause. This concept has given rise to the "double hit hypothesis", which proposed that PD may result from an interaction between multiple genetic mutations and/or the combination of a mutant gene and an environmental toxin. [445] In support of this concept, Good *et. al.* [215] have shown that there is subclinical nigral degeneration in the SOD-mutant mouse and that dopamine neurons in this mouse are highly sensitive to small doses of MPTP that do not affect wild-type littermates. Although it is unlikely that an α-synuclein mutation will account for many cases of PD, this discovery may permit the development of a transgenic animal and provide an opportunity to better understand the mechanism of cell death in PD.

Immunological mechanisms

Microglia and cytokines are known to mediate cellular responses after injury to the CNS. Large numbers of reactive (HLA-DR) positive microglia have been observed in the SN in PD, particularly in areas of maximal neurodegeneration, namely the ventral and lateral portions of the SN. Levels of interleukin-1β (IL-1β), interferon-α (INF-α), and tumor necrosis factor-α (TNF-α) in the SN of PD patients are increased by 760–1570% in comparison with normal controls. TNF- α is particularly increased in PD in the region of neuromelanin-containing neurons or debris. Activation of TNF-α receptors is associated with nuclear translocation of nuclear factor b kappa (NF-κB) and with the development of apoptosis in cultured dopaminergic neurons. [275] Interestingly, NF-κB translocation and apoptosis are preceded by the transient production of free radicals. These events can be prevented by the antioxidant N-acetylcysteine, indicating that an oxidant-mediated apoptogenic transduction pathway may play a role in neuronal death. In PD patients, there is a 70-fold increase in nuclear translocation of NF-κB. This suggests that activation of TNF-α and similar transduction mechanisms may play a similar role in PD neurodegeneration. [445]

It remains unclear whether an immune/inflammatory component is a primary or secondary event in PD or whether changes observed are due to an autoimmune process or to the natural response of microglia and astroglia to neuronal damage. However, it is becoming increasingly likely that glia may play some role in neurodegenerative conditions. A greater understanding of the role of glial cells and their regulation of neuroactive molecules might nonetheless contribute to the design of therapies that will protect or repair degenerating nerve cells.

Oxidative Stress

Oxidative stress has received the most attention in PD because of the potential of the oxidative metabolism of dopamine to yield hydrogen peroxide (H_2O_2) and other ROS. [442] Oxidant stress and consequent cell death could develop in the SN under circumstances in which there is (1) increased dopamine turnover, resulting in excess peroxide formation; (2) a deficiency in glutathione (GSH), thereby diminishing the brain's capacity to clear H_2O_2; or (3) an increase in reactive iron, which can promote $OH^{\cdot}$ formation. Indeed, postmortem studies of PD brains demonstrated increased iron, decreased GSH, and oxidative damage to lipids, proteins, and DNA, suggesting that the SNc is in a state of oxidant stress. [304]

There is evidence of oxidative damage in the brains of PD patients. Increased levels of the lipid peroxidation products malondialdehyde (MDA) and lipid hydroperoxide have been found in the SNc but not the cerebellum of PD patients. [144] Increased staining for 4-hydroxynonenal, a product of lipid peroxidation that has the capacity to alter proteins and promote cell toxicity, has been detected in surviving dopaminergic neurons. Additionally, increased levels of protein carbonyls and 8-hydroxy-2-deoxyguanosine, reflecting oxidative damage to proteins and DNA, respectively, have been found in the SNc as well as in numerous other brain regions of PD patients. [8, 9] Overall, these results suggest that there is widespread oxidative damage in PD. It remains to be determined if the oxidative damage that occurs in PD is a primary event or occurs secondarily to an alternative etiology, drugs, or postmortem events. [14]

Mitochondrial Dysfunction

A number of studies now suggest that a selective 30–40% decrease in complex I activity of the mitochondrial respiratory chain occurs in the SNc of PD patients. [145, 509, 510] Other brain regions are unaffected, and a similar defect has not been found in patients with multiple system atrophy (MSA) who have also experienced extensive degeneration of nigrostriatal neurons and been exposed to levodopa. A complex I defect has also been found in platelets and muscle of PD patients, but these results are less consistent, especially in muscle. [493] The cause of the decreased complex I activity in PD remains a mystery. MPTP-like toxins have not been detected. No specific abnormality has been detected in the subunits of complex I or in the mitochondrial or nuclear genes that encode complex I proteins.

A mitochondrial complex I defect could contribute to cell degeneration in PD through decreased ATP synthesis and a bioenergetic defect. In mouse brain synaptosomes, complex I inhibition by MPTP or MPP^+ can lead to depletion of cellular ATP. [411] However, studies in experimental animals indicate that a decrease in complex I activity of 40% or less does not compromise cellular ATP levels. A decrease in α-ketoglutarate dehydrogenase (α-KG) immunostaining has also been detected in PD. The combination of a decrease in both α-KG and complex I activity would be more likely to adversely affect cellular energy metabolism than would a defect in either enzyme alone. [407]

A mitochondrial complex I defect could also lead to cell damage through free radicals generated directly at this site or by way of a compensatory increase in respiration at complex II. It is noteworthy that MPTP toxicity can be attenuated by free radical scavengers and by coenzyme Q, a redox component of the mitochondrial respiratory chain that accepts electrons from complex I or II. [514] A complex I defect might also contribute to the development of apoptosis. Increasing evidence suggests that a reduction in the mitochondrial membrane potential as a result of impaired proton pumping can lead to opening of a mitochondrial permeability transition pore and the release of small mitochondrial proteins that signal the onset of apoptosis. As complex I is the major site of proton pumping, it is possible that a complex I defect in PD may contribute to neuronal vulnerability and thus lead to apoptosis.

Neurotrophic Factors

Target-derived trophic factors are substances which are produced and released in limited quantities in the projection areas of neurons which respond to them. Within the nervous system, the primary effect of these trophic factors is on survival and differentiation of neurons which respond to them. Classic *in vitro* studies showed that cultured sympathetic neurons could not survive if deprived of nerve growth factor (NGF). [445] Similarly, axotomy can induce degeneration of nigrostriatal dopamine neurons by depriving them of access to essential survival factors in target tissues. It is also apparent that both nerve cells and astrocytes can synthesize mRNAs and protein of neurotrophic molecules, including ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF). These factors have the capacity to support the survival of neighboring nerve cells. [198] In the normal adult central nervous system, these trophic factors are constitutively expressed at low levels, but they can be up-regulated following injury. Sublethal neuronal damage in adult rats induces reactive astrocytes with up-regulation of neurotrophic factors such as CNTF, NGF, and fibroblast growth factor (FGF). Reactive astrocytes have been demonstrated in regions of dopaminergic nerve cell loss in PD. There is also strong evidence that a number of trophic molecules have the capacity to protect dopamine neurons from toxic insults. BDNF increases survival of cultured dopaminergic neurons and protects them from exposure to MPTP. Both GDNF and CNTF protect SN neurons in rats from transection of nigrostriatal axons. GDNF has been shown to increase the survival and sprouting of dopaminergic neurons in dopamine-lesioned rodents and primates and to reverse Parkinsonian features in MPTP-treated primates. [631] Both neuroprotective and neurorestorative benefits have been seen. An *in situ* hybridization study found no detectable levels of GDNF mRNA in brains obtained from PD patients or age-matched controls. It would seem likely, therefore, that a decline in GDNF expression does not initiate dopaminergic cell loss. However, a reduction in the capacity to up-regulate trophic factors in response to injury might deprive the cells of an important defense mechanism and contribute to cell degeneration. Alternatively, administration of trophic factors may rescue or protect dopamine neurons. Systemic delivery of trophic factors is limited in CNS penetrance, and effective therapy in animal models requires direct intracerebral or intrathecal delivery. A small trial of intraventricular GDNF has been initiated in PD patients, but results are not yet available. Other possible approaches include the use of implanted or encapsulated cells that express trophic factors or adenovirus vectors that transfect cells in the SNc or striatum to produce a trophic factor. Preliminary studies with GDNF administrated by each of these methods have been shown to protect dopamine cells in rodents, illustrating their potential value in PD. [204, 235]

Normal aging process

Normal aging is associated with clinical features somewhat reminiscent of PD, including the assumption of a stooped posture, slowing of body movement and a reduction of associated movements. Aging is associated with a linear decline of pigmented neurons in SN [539] and with a decreased level of striatal dopamine, TOH, and dopa decarboxylase. This has led to the suggestion that PD may result from the effects of aging superimposed upon an insult to the SN earlier in life. This may be unrecognized, as symptoms of Parkinsonism do not become apparent until DA depletion exceeds 50%. However, the pattern of the age related SN dopamine depletion is different from PD, [321] and the presence of activated microglia at all stages of PD suggests that there is an ongoing process with aging. [94, 587]

Other relevant factors

Apoptosis is a factor involved in PD, as it is in other neurodegenerative diseases. How this programmed cell death progresses in PD is not very well understood. MPTP-induced apoptotic cell death has been observed in cell culture. [73] A recent observation suggests that apoptosis in PD is trigged by oxidative stress, and it is somehow related to NF-κB pathways. [275]

The role of other factors which determine the fate of dopaminergic neurons during the pathogenesis of PD is not fully understood. One such factor is *sonic hedgehog*, a protein which is expressed in notochord and plant cells and induces a ventral cell phenotype. [538] The weaver mutation is a spontaneously arising defect in mice, characterized by degeneration of cerebellar granules and midbrain DA neurons. It has been linked to a mutation of G-protein-gated inward rectifying potassium channels. Further details on the mechanisms of SN cell death are not yet understood.

1.1.2.3.2.5. Treatment of PD

Based on current knowledge regarding the etiology, pathogenesis, and mechanism of cell death in PD, numerous neuroprotective strategies may be devised. The treatment plan can be very complicated, because different environmental and genetic factors likely contribute to the development of PD and multiple causes may be operative even in an individual patient. Neuroprotection may be provided by agents that interfere with factors involved in pathogenesis. These may include antioxidants, bioenergetics, agents that interfere with excitotoxicity or prevent a rise in cytosolic free calcium, trophic factors, and anti-inflammatory drugs. If apoptosis is taken into consideration in the treatment of PD, there is an opportunity to interfere with neuronal death. Treatments involving the effector stages of apoptosis may have the advantage of providing benefit to patients regardless of the specific etiology or pathogenesis of PD. Delineation of the relationship between inhibition of MAO-B oxidation, NMDA receptor antagonism, and apoptosis will hopefully point the way to the development of newer, more potent agents that can slow the progression of PD by protecting vulnerable neurons and reducing the death of dopaminergic neurons.[424, 425, 444] Moreover, surgery is one of the strategies in PD treatment. Thalamotomy effectively reduces tremor and sometimes rigidity on the contralateral side. Thalamic stimulation can reproduce the benefits of thalamotomy without the risk of irreversible tissue loss, because no physical lesion is created. Pallidotomy is a procedure in which a portion of the globus pallidus is lesioned permanently. Compared with thalamic procedures, pallidotomy is less beneficial for tremors and more beneficial for dyskinesias.

A brain tissue transplant is also one of the options in treatment. Although there have been encouraging results, transplantation surgeries are still in the experimental stage. The experiments began with fetal tissue, but now scientists are also working with genetically engineered cells and a variety of animal cells that can be made to produce dopamine. Deep brain stimulation is a similar procedure to pallidotomy. This technique also seeks to stop uncontrollable movements. It is based on the technology of cardiac pacemakers. Electrodes are implanted in the thalamus or globus pallidus and connected to a pacemaker-like device, which the patient can switch on or off as symptoms dictate. [1]

1.1.2.3.3. Other neurodegenerative diseases

As discussed in the above sections concerning AD, PD, and other degenerative diseases, neurodegenerative disorders are a heterogeneous group of diseases of the nervous system that have many different etiologies. These diseases that we have not discussed include Lewy body diseases, Multi-infarct dementia (MID), Pick's disease, Huntington's disease, Amyotrophic lateral sclerosis (ALS), Creutzfeldt-Jakob disease (CJD), Frontal lobe degeneration (FLD), Corticobasal degeneration (CBD), Multiple system atrophy (MSA), Progressive supranuclear palsy (PSP), etc. Many are hereditary, some are secondary to toxic or metabolic processes, and others result from infections. Many of these diseases have no known etiology. The neurodegenerative diseases are often age associated, chronic and progressive without known treatment modalities. Neuropathologically, these diseases are characterized by abnormalities of relatively specific regions of the brain and populations of neurons. These cell groups in the different diseases determine the clinical phenotype of the illnesses. Due to the prevalence, morbidity and mortality of the neurodegenerative diseases, they represent significant medical, social, and financial burdens. Recent investigations in medical genetics have identified specific genes for several neurodegenerative disorders and animal models have begun to be used to study the etiological factors and pathogenic mechanisms. These in turn may lead to new therapeutic approaches. [71]

1.2. Cell death (apoptosis & necrosis) in neurodegeneration

1.2.1. Introduction of apoptosis

Kerr *et al* [316, 317] first coined the term apoptosis in 1971. This Greek word meaning leaves falling from a tree describes an intrinsic cell suicide program involved in the normal turnover of hepatocytes. Cell morphologic manifestations of apoptosis include condensation of cell and nuclear contents, nuclear membrane breakdown, and the formation of apoptotic bodies that are small membrane-bound vesicles phagocytosed by neighboring cells. Molecular components of the apoptotic pathway were first described in two important studies. Genetic studies in *Caenorhabditis elegans* revealed three genes, *ced3, ced4,* and *ced9,* that specifically function in a pathway that controls developmental specific cell death. [4, 166] Second, *Bcl-2,* a human oncogene overexpressed in follicular lymphoma, was found to influence cell apoptotic response. These discoveries ignited an explosion of research into apoptosis that in the past decade has unveiled a complex, yet cohesive, picture of this intrinsic cell suicide program. [4] Apoptotic signals, both intracellular and extracellular, converge to activate a group of apoptotic-specific cysteine proteases termed caspases that cleave their substrates with signature specificity after aspartic acid residues. [556] Chromatin condensation, DNA fragmentation into nucleosomal fragments, nuclear membrane breakdown, and the formation of apoptotic bodies are direct consequences of caspase activity (Fig. 1.3).

Cell death via apoptosis is a prominent feature in mammalian neural development. Recent studies into the basic mechanism of apoptosis have revealed biochemical pathways that control and execute apoptosis in mammalian cells. Protein factors in these pathways play important roles during development in regulating the balance between neuronal life and death. Additionally, mounting evidence indicates such pathways may also be activated during several neurodegenerative diseases, resulting in improper loss of neurons.

1.2.2. Biochemical mechanisms of apoptosis

1.2.2.1. DNA fragmentation and chromatin condensation during apoptosis

The fragmentation of DNA into nucleosomal fragments was one of the first cellular features of apoptosis to be identified. It is commonly used as a biochemical marker for apoptosis. [612] *In situ*, nucleosomal DNA fragmentation is assayed by the TUNEL (TdT-mediated dUTP-biotin nick end labeling) stain. Free DNA ends are end labeled with biotinylated poly-dUTP by terminal deoxytransferase and then stained using avidin-conjugated peroxidase. [277] DNA fragmentation is mediated by a heterodimeric factor of 40 and 45 kDa, respectively, in human [DNA fragmentation factor (DFF) 40 and 45] and in mice [caspase activated DNase (CAD) and inhibitor of caspase-activated DNase (ICAD)]. DFF40/CAD and DFF45/ICAD are encoded by novel genes and do not share sequence homology with other proteins with known functions. In apoptotic cells, DFF45, which has two caspase cleavage sites, is cleaved into three smaller fragments.

Cleaved DFF45 dissociates from DFF40, inducing oligomerization of DFF40 into a large protein complex that has DNase activty. [356] DFF activity can only be reconstituted by coexpressing the two subunits. [168] When expressed alone, DFF40 has lower expression and no DNase activity, which suggests that DFF45 functions as a specific molecular chaperone important for DFF40 activation and synthesis. Unlike other DNases, DFF40 is significantly stimulated by internucleosomal, chromatin-associated proteins such as high mobility group (HMG)-1, -2, and -14 and histone H1 but not core histones. HMGs and histone H1 may target DFF40 to the internucleosomal linker region, resulting in the exquisite pattern of internucleosomal DNA fragmentation commonly detected during apoptosis. The multimeric nature of the active DFF40 may also contribute to apoptotic chromatin condensation by pulling cleaved nucleosomal fragments together. After treatment with active DFF40, nuclei stained with a DNA dye exhibit bright particles, an apoptotic hallmark indicative of chromatin condensation. [354] Thymocytes and splenocytes from mice deficient in ICAD die by apoptosis but fail to condense chromatin or fragment DNA. ICAD null mice develop normally and are fertile, indicating that DNA fragmentation and chromatin condensation during apoptosis are not essential for normal development of a mouse. [628]

1.2.2.2. Caspase activation pathways

In living cells, caspases exist as inactive zymogens that, like DFF, are activated by proteolytic cleavage. [555] There are two relatively well-studied pathways that lead to caspase activation. One pathway involves death receptors, such as Fas, and a tumor necrosis factor (TNF) receptor at the cell surface, leading to the intracellular activation of caspase-8. [492] Fas ligand and TNF, which usually exist as trimers, bind and activate their receptors by inducing receptor trimerization. Activated receptors recruit adaptor molecules such as FADD/MORT1 (Fas-associating protein with death domain), which recruit procaspase-8 to the receptor complex, where it undergoes autocatalytic activation. [580, 581] Activated caspase-8 will cleave and activate other downstream caspases, such as caspase-3, caspase-6, and caspase-7, constituting the main caspase activity of apoptotic cells. Another means of caspase activation is through the release of cytochrome *c* from the mitochondria. Cytochrome *c* is a 13-kDa soluble electron transfer protein exclusively located in the mitochondrial intermembrane space. During apoptosis, however, the outer membrane of mitochondria becomes permeable to cytochrome *c*, which binds to Apaf-1. [353]

Apaf-1 is a 130-kDa cytosolic monomer consisting of three distinctive domains: a caspase recruitment domain, a CED4 homologous domain, and a series of WD40 repeats. [633] On induction of apoptosis, Apaf-1 forms a multimeric complex with cytochrome *c*. Apaf-1/ cytochrome *c* complexes are sufficient to recruit and activate procaspase-9. Activated caspase-9 released from the complex activates downstream caspases such as caspase-3, caspase-6, and caspase-7. [633]

1.2.2.3. Regulation of cytochrome *c* release by the *Bcl-2* family of proteins

A major regulatory step for caspase activation is at the level of cytochrome *c* release from mitochondria to the cytosol. Cytochrome *c* release initiates caspase activation by activating Apaf-1. It also breaks the electron transfer chain, resulting in reduced energy generation and more ROS due to incomplete reduction of atomic oxygen.

[220] The release of cytochrome *c* is regulated by the *Bcl-2* family of proteins, including anti-apoptotic members *Bcl-2* and *Bcl-x_L* and pro-apoptotic *Bak, Bim, Bad,* and *Bax.* [4] Overexpression of anti-apoptotic *Bcl-2*, or its close homologue *Bcl-x_L*, blocks cytochrome *c* release induced by a variety of apoptotic stimuli. [318] In contrast, *Bax*, *Bak*, and *Bid* have been shown to directly cause cytochrome *c* release both *in vivo* and *in vitro*. [336] The mechanism of cytochrome *c* release and its regulation by the *Bcl-2* family of proteins is not known. One possibility is that changes in mitochondrial membrane permeability induce mitochondrial swelling, causing outer membrane rupture. [344] On the other hand, increases in outer membrane permeability may occur independent of swelling.

Intrinsic or extrinsic death signals may be transmitted to the mitochondria by the translocation of pro-apoptotic *Bcl-2* family members to the mitochondria from different cellular compartments. Extracellular death signals such as Fas ligand or TNF activate caspase-8 intracellularly. Activated caspase-8 cleaves and activates Bid, which translocates to the mitochondria and induces cytochrome *c* release, amplifying the caspase activation signal. [48] Extracellular survival signals inhibit apoptosis by activating the phosphotidylinositol 3 kinase/Akt pathway, leading to *Bad* phosphorylation. Phosphorylated *Bad* binds 14–3-3 protein and is sequestered in the cytoplasm, whereas dephosphorylated *Bad* translocates to the mitochondria. Conversely, Ca^{++} may induce apoptosis by activating the calcineurin-dependent phosphatase that dephosphorylates *Bad*. Other intrinsic death signals may regulate *Bim* translocation. In normal living cells, *Bim*, a pro-apoptotic *Bcl-2* family member, binds to LC8, a cytoskeletal component. After cells are induced to die by apoptosis, the *Bim*/LC8 complex dissociates from the cytoskeleton and translocates to the mitochondria. *Bax* has also been shown to translocate from the cytoplasm to the mitochondria during apoptosis.[482] Apoptotic signals may activate the translocation of these factors to the mitochondria, which then trigger cytochrome *c* release, inducing caspase activation.

1.2.3. Apoptosis in neurodegeneration

Gradual loss of specific types of neurons from different parts of the CNS characterizes the pathological progression of a variety of neurodegenerative disorders.[273] A site of intense apoptosis is present in the CNS during development (some estimate 50%-80% of CNS neurons die during development) and appears to depend on the expression of survival-promoting genes like *Bcl-x_L* for survival in adulthood. It may be especially vulnerable to derangement of apoptotic pathways, particularly pathways involving calcium and free-radical generation. Apoptotic cell death and its attendant molecular mediators appear to play a role in many neurodegenerative disorders, such as AD, PD, and many others.[301] Progressive cell loss in specific neuronal populations is the pathological hallmark of neurodegenerative diseases, but its mechanisms remain unresolved. However, DNA fragmentation in the human brain as a sign of neuronal cell injury is too frequent to account for the continuous loss in these slowly progressive diseases. In a series of autopsy confirmed cases of AD, PD, related disorders, and age-matched controls, DNA fragmentation using the TUNEL method, an array of apoptosis-related proteins (ARP), proto-oncogenes, and activated caspase-3, (the key enzyme of late-stage apoptosis), were examined.[301] In AD, a considerable number of hippocampal neurons and glial cells showed DNA fragmentation, with a 3- to 6-fold increase related to neurofibrillary tangles and amyloid deposits. However, only 1 in 2.6 to 5.6 neurons displayed apoptotic morphology and cytoplasmic immunoreactivity for activated caspase-3, whereas no neurons were labeled in age-matched controls. Caspase-3 immunoreactivity was seen in granules of cells with granulovacuolar degeneration, with around 25% co-localized with early cytoplasmic deposition of *tau*-protein. [301] In PSP, only single neurons and several oligodendrocytes in the brainstem, some with *tau*-deposits, were TUNEL-positive and expressed both ARPs and activated caspase-3. In PD, dementia with Lewy bodies, MSA, and corticobasal degeneration, TUNEL-positivity and expression of ARPs or activated caspase-3 were only seen in microglia and oligodendrocytes with cytoplasmic inclusions, but not in neurons. These data provide evidence for extremely rare apoptotic neuronal death in AD and PSP. This is compatible with the progression of neuronal degeneration in these chronic diseases. Apoptosis mainly involves reactive microglia and oligodendroglia, the latter often exhibiting deposits of insoluble fibrillary proteins, while alternative mechanisms of neuronal death may occur. Susceptible cell populations in a proapoptotic environment show increased vulnerability towards metabolic or other noxious factors, with autophagy as a possible protective mechanism in the early stages of programmed cell death. The intracellular cascade leading to cell death still awaits elucidation.

Mutations in the PS2 gene have recently been associated with familial AD. [485] The PS2 is hypothesized to function in an apoptosis pathway downstream of Fas. PS2's mouse homologue prevents the up-regulation of Fas on T lymphocytes, which normally occurs when the T cell is activated by binding to a foreign peptide. In the PC12 neuronal cell line, overexpression of normal PS2 leads to apoptosis. A mutant PS2 isolated from patients with familial AD has an even greater ability to induce apoptosis. One of the cardinal features of AD is formation of amyloid plaques in the brain. The existence of these plaques also alters the apoptotic threshold of neurons [357]. In primary cultures of human neurons, peptide fragments of amyloid can down-regulate anti-apoptotic *Bcl-2* and up-regulate proapoptotic *Bax* expression, thus making the neurons more prone to die, especially in response to oxidative stress. [289]

PD is characterized by the degeneration of nigrostriatal dopaminergic neurons, which are thought to die by apoptosis and necrosis in response to oxidative damage. [558] Although predisposing apoptotic genes for PD have been more elusive than those for AD, the treatment for PD has turned out to be intimately involved in the inhibition of apoptosis. The drug selegiline hydrochloride has been used historically as a treatment for PD because of its ability to irreversibly inhibit MAO-B, thus enhancing dopamine signaling. The effect of selegiline on MAO-B may be of less importance than the recent discovery that it can specifically alter transcription of cellular death and survival genes, including superoxide dismutases, *Bcl-2* and *Bcl-*x_L, NOS, and nicotinamide adenine dinucleotide dehydrogenase. [444] Selegiline prevents the progressive reduction in mitochondrial membrane potential and thus may inhibit the release of proapoptotic substances from the mitochondria.

Much evidence has been gathered implicating apoptosis in neurodegenerative diseases. Although these studies provide hope that the apoptotic program is an effective therapeutic target, important questions about how the diseases induce apoptosis remain. Is apoptosis the cell's reaction to a permanent neuronal insult inflicted by disease or is the disease directly involved in activating the apoptotic program? In the latter case, anti-apoptotic agents such as caspase inhibitors could be used to inhibit cell death and preserve cellular integrity. In the former case, however, caspase inhibitors would be less beneficial because cellular integrity would already be compromised by disease. AD, PD and other diseases are associated with intracellular or extracellular protein aggregation. Recent reports suggest that neurodegeneration may be the result of protein aggregation that directly activates caspases and induces apoptosis. It is difficult to imagine how mutated disease genes might directly activate the apoptotic program, because disease onset occurs in later life. However, the formation of aggregates later in life might serve as the "rate-limiting step" in triggering the degenerative disease process. The recruitment of apoptogenic proteins to protein aggregates may activate apoptotic pathways because oligomerization steps are required for the formation of active multimeric complexes in FADD/caspase-8, Apaf-1/caspase-9, and DFF pathways. In such cases, drugs that prevent aggregate formation or inhibit caspase activation might be effective therapies.

A putative pathway of neuronal apoptosis has been summarized and shown in Fig. 1.3.

1.2.4. Anti-apoptotic signaling and neuronal life

The nervous system deploys a wonderful array of signaling mechanisms designed to prevent neuronal apoptosis. [372] The most intensively studied of such anti-apoptotic signaling pathways are those activated by neurotrophic factors and cytokines. Neurotrophins (NGF, BDNF, neurotrophins 3 and 4/5), basic fibroblast growth factor (bFGF), and insulin- like growth factor-1 (IGF-1) activate receptors with intrinsic tyrosine kinase activity. It has been found that each of these neurotrophic factors can protect cultured neurons against various apoptotic insults (e.g., oxidative, metabolic, and excitotoxic insults) by mechanisms involving stabilization of cellular calcium homeostasis and suppression of oxyradical production [108, 368, 369, 371] (Fig.1.4., NGF as an example). The neurotrophic factors thereby suppress the apoptotic process at an early step, prior to mitochondrial dysfunction and caspase activation [375]. At least a component of the anti-apoptotic mechanism of action of neurotrophic factors involves modulation of expression of genes that encode proteins involved in calcium regulation and free radical metabolism (e.g., antioxidant enzymes). [244] The cytokine TNF-α, named for its ability to induce death in some types of tumor cells, can prevent neuronal excitotoxicity and apoptosis. For example, pretreatment of cultured rat hippocampal neurons with TNF-α results in increased resistance to excitotoxicity [107] and apoptosis induced by exposure to Aβ or iron. [26, 381] TNF-α also protects cultured neurons against apoptosis induced by trophic factor withdrawal. Endogenous, injury-induced TNF-α appears to serve a neuroprotective role because excitotoxic and ischemic neuronal injury is increased in mice lacking TNF-α receptors. [83] Work aimed at understanding the mechanism whereby TNF-α prevents neuronal apoptosis led to the discovery that activation of the transcription factor NF-κB serves an important anti-apoptotic role in neurons. NF-κB is localized in the cytoplasm in an inducible form consisting of the transcription factor dimer (p50–p65) and an associated inhibitory subunit called I-κB. Activation of NF-κB occurs when I-κB is phosphorylated and dissociates from the p50–p65 dimer; the dimer then translocates to the nucleus and binds regulatory elements in κB responsive genes. TNF-α induces NF-κB activation in cultured neurons, and several manipulations that more directly activate NF-κB (e.g., suppression of I-κB expression or treatment of cells with C2-ceramide) mimic the excitoprotective and anti-apoptotic actions of TNF-α. [25, 219] Activation of NF-κB is required for the neuroprotective actions of TNF-α because treatment of cells with κB decoy DNA abolishes the protective effect of TNF. Neurons exhibit a constitutive level of NF-κB activity, and suppression of this basal activity enhances neuronal apoptosis. The gene targets regulated by NF-κB that may mediate its anti-apoptotic actions include those encoding the antioxidant enzyme manganese superoxide dismutase, the calcium-binding protein calbindin D28k, [108] and glutamate receptor subunits. [189, 190] In addition to being activated by TNF-α, NF-κB is activated in response to the secreted form of amyloid precursor protein and oxidative stressors. [243]

In addition to the neuroprotective actions of intercellular signals such as neurotrophic factors and cytokines, intrinsic cytoprotective signaling mechanisms are activated when neurons are subjected to stress. Increased levels of intracellular calcium $[Ca^{++}]_i$, such as occur following overactivation of glutamate receptors, trigger several different protective mechanisms. For example, calcium induces activation of transcription factors that are also activated by neurotrophic factors and TNF-α, including activating protein-1 (AP-1) and NF-κB. Calcium activates gelsolin, a protein that cleaves actin filaments. Actin depolymerization in turn modulates the activity of voltage-dependent calcium channels and *N*-methyl-D-aspartate (NMDA) receptors so that the channels are harder to open, resulting in less calcium influx. [187, 188] Increased levels of oxidative stress in neurons result in activation of NF-κB and increased production of cyclic GMP. [24, 387]

Cyclic GMP has been shown to protect neurons against excitotoxicity by inducing activation of high-conductance, charybdotoxin-sensitive potassium channels. [186, 191] Increased levels of oxidative and metabolic stress also induce expression of "stress" proteins such as heat-shock protein-70 (HSP-70) and glucose-regulated protein-78 (GRP-

78). Each of these stress proteins has been shown to protect neurons against excitotoxic and apoptotic insults.[622, 623]

1.2.5. Necrosis or apoptosis in neurodegeneration

Severe neuronal loss is common to many neurodegenerative diseases. Although there is now increasing evidence to show that apoptosis can significantly contribute to neuronal demise in neurodegenerative diseases, necrotic features are often prevalent in neuropathological conditions. For instance, in AD, amyloid formation may very well be at the end of a pathophysiological cascade, set in motion by many different triggers. This cascade could involve excessive apoptosis, followed by necrosis and inflammation.[150, 441] PD is an idiopathic disease caused by necrosis and apoptosis of dopaminergic cells in the brainstem, probably induced by oxidative stress.[32] Both acute and chronic insults to the nervous system can result in changes in homeostasis that lead to cell death or recovery processes that alter function. The signaling mechanisms for this broad spectrum of events that impair neurological function range from abrupt injury to the slow onset of neurodegenerative diseases in extreme aging. A common element in all of these events is the triggering of signal cascades that determine cellular commitment to apoptosis as an ameliorative alternative to inflammatory necrosis. In addition, metabolic defects leading to ATP depletion can consequently switch execution of cell death towards necrosis.[435] Mitochondria have been linked to both necrotic and apoptotic cell death.[328] Mitochondrial dysfunction has been observed to occur in many neurodegenerative diseases, and this dysfunction is thought to have a major role in the pathogenesis of these diseases.[48] Nevertheless, it is unclear which of the two types of demise, apoptosis or necrosis, prevails in acute and slowly developing neurogenerative disorders, and whether the mode of cell death is relevant for the ultimate progression of the disease.

In many neurodegenerative diseases, it is likely that cell death does play a primary role in loss of function, although it has not been proven. For example, it remains a strong possibility that synaptic injury precedes neuronal injury in AD. Assuming a primary, or even a secondary role for cell death in degenerating nervous system diseases, there is suggestive evidence from a variety of conditions, mostly based on *in vitro* models, that some of the neuronal death occurs via apoptosis. During apoptosis, there is activation of complicated interlocking sets of pathways. Whether intervening in the cascade of programmed cell death, or even totally halting apoptosis, would have any significant impact on the signs or symptoms of the diseases is currently unknown. It may be that apoptosis itself is a late phenomenon, and that the signals or stresses leading to neuronal apoptosis are themselves the earlier cause of irreversible neuronal dysfunction.[282] However, continued investigation into the cell pathways involved in apoptosis can increase our understanding of the pathological changes in diseases, and can lead to therapeutic plans.

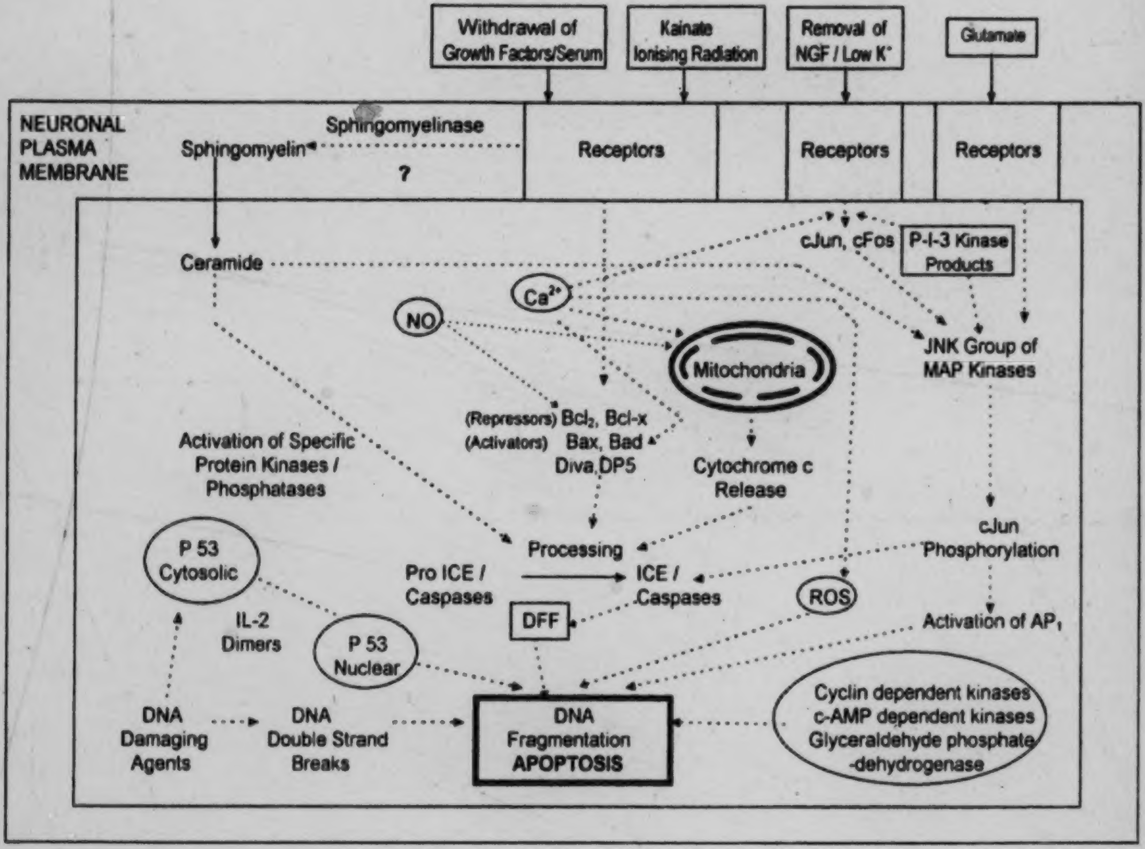

Withdrawal of Growth Factors/Serum
Kainate Ionising Radiation
Removal of NGF / Low K⁺
Glutamate
NEURONAL PLASMA MEMBRANE
Sphingomyelinase
Sphingomyelin
?
Receptors
Receptors
Receptors
cJun, cFos
P-I-3 Kinase Products
Ceramide
Ca²⁺
NO
Mitochondria
JNK Group of MAP Kinases
(Repressors) Bcl₂, Bcl-x
(Activators) Bax, Bad
Diva,DP5
Cytochrome c Release
Activation of Specific Protein Kinases / Phosphatases
P 53 Cytosolic
Processing
cJun Phosphorylation
Pro ICE / Caspases
ICE / Caspases
ROS
IL-2 Dimers
DFF
Activation of AP₁
P 53 Nuclear
Cyclin dependent kinases
c-AMP dependent kinases
Glyceraldehyde phosphate -dehydrogenase
DNA Damaging Agents
DNA Double Strand Breaks
DNA Fragmentation APOPTOSIS

Fig. 1.3. Putative pathways for apoptosis in the nervous system (modified from [501]).

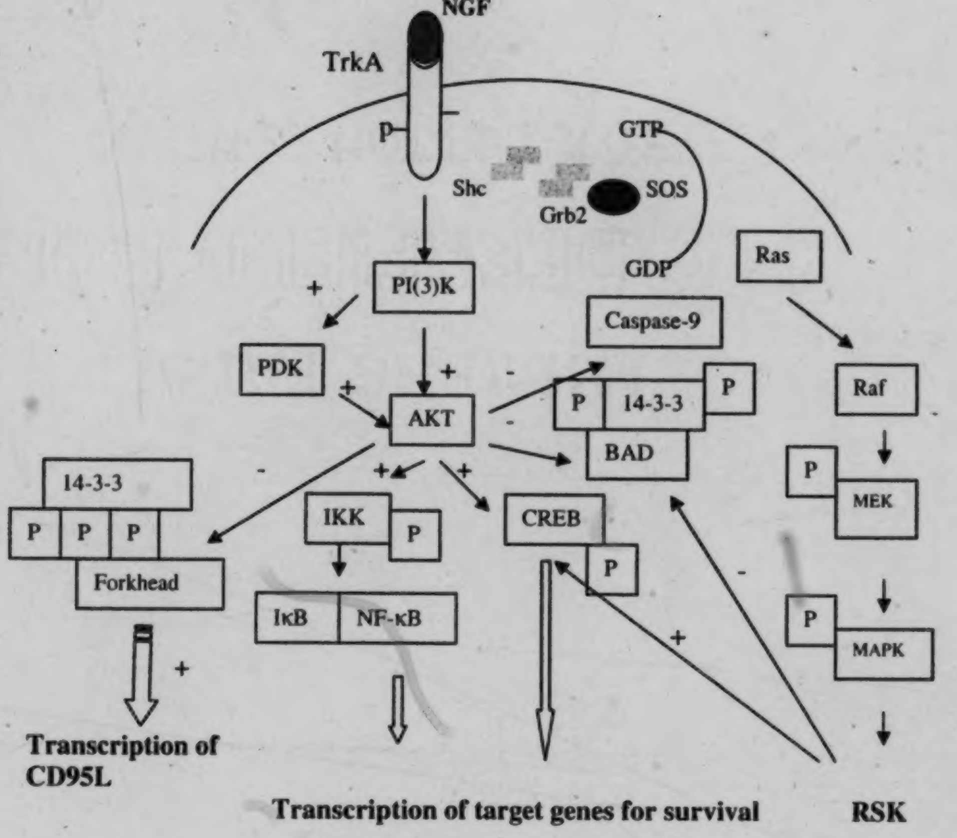

NGF
TrkA
P
Shc
Grb2
SOS
GTP
GDP
Ras
PI(3)K
PDK
AKT
Caspase-9
P
14-3-3
P
BAD
Raf
P
MEK
P
MAPK
14-3-3
P
P
P
Forkhead
IKK
P
IκB
NF-κB
CREB
P
+
-
Transcription of CD95L
Transcription of target genes for survival
RSK

Fig. 1.4. Neuronal survival pathways induced by the binding of NGF to its receptor TrkA.

NGF induces the autophosphorylation of TrkA which provides docking sites for signal transduction molecules such as phospholipase C, phosphoinositide 3-kinase (PI(3)K) and the adaptor protein Shc. Activated PI(3)K induces the activation of Akt through 3'-phosphorylated phosphatidylinositol as well as phosphoinositide-dependent kinase (PDK), which in turn phosphorylates and activates Akt. The phosphorylation of CREB and IKK stimulates the transcription of pro-survival factors; whereas the phosphorylation of *Bad*, Forkhead and caspase-9 inhibits the pro-apoptotic pathway. In a parallel pathway, the interaction of Shc–Grb2 and SOS activates the Ras–Raf–MEK–ERK pathway, resulting in the activation of Rsk. *Bad* and CREB are also the targets of Rsk that might act synergistically with Akt to activate the survival pathway (modified from [624]).

1.3. The neuroprotective effects of estrogen

It is well recognized that estrogen regulates sex differentiation and sex organ maturation via binding to specific intracellular receptors. However, estrogen receptors (ERs) are expressed in a variety of other tissues, including the nervous system, suggesting that estrogen's effects are not limited to primary and secondary sex organs. Increasing evidence supports the role of estrogen as a neuroprotective compound that can act dependently or independently of ER activation. Estrogen has recently been shown to exhibit intrinsic antioxidant activity that is ER independent. [39] Clinical studies suggest that estrogens influence aspects of memory and cognition, postural stability, balance, movement and fine motor skills, and mood. Furthermore, the results of these studies emphasize that estrogens exert protective actions against neurodegenerative diseases and brain injury. Studies performed with laboratory animals confirm many of these observations and have increased our understanding of possible mechanisms. Together, these studies clearly establish that estrogens are pleiotropic hormones with multiple targets in the brain and that these complex actions are potentially mediated by diverse, multifactorial, and interacting mechanisms of action. Thus, estrogen might represent a potential "chemical shield" for neurons. [606]

During this century, the average life span of women has increased dramatically from approximately 50 years to over 80 years of age. During this interval, the time of menopause has remained essentially fixed at approximately 51 years of age. [453] This profound physiological change is accompanied by a dramatic decrease in estrogen levels. Thus, an increasing number of women are living a larger portion of their lives in a chronically hypoestrogenic state. The knowledge that estrogen exerts protective and trophic effects on the brain, together with information on extended life expectancy, lead to the logical conclusion that postmenopausal women may be more vulnerable to neurological diseases and injury and may suffer increasingly from neural dysfunction as they age. Thus, it is critical that we should understand better the importance of estradiol in maintaining normal brain function in the face of aging and injury.

In this section, observations and mechanisms of estrogen as a neuroprotectant will be discussed.

1.3.1. Neuroprotective action of estrogen

Estrogen exerts multiple and diverse actions on the brain during development. During fetal development and the early postnatal period, estrogen is a potent trophic factor that influences brain development and differentiation, cell survival, and plasticity. It exerts these actions by mediating neurogenesis, [194] cell death, [15, 396] cell migration, [80] neuronal somatic and dendritic growth, [544] synapse formation and elimination, [288] and glial differentiation and neuronal morphology. [195, 196, 402] Several investigators have found that addition of estrogen *in vitro* to organotypic cultures or dispersed cells derived from embryonic or neonatal rodent hippocampus, cerebral cortex, or hypothalamus elicits a striking enhancement of neurite outgrowth, extensive arborization of their branches, and synaptogenesis. [50, 563] Estrogen may mediate synapse formation and plasticity during development and throughout adult life by modulating trophic factors and their receptors [195, 477] or influencing other molecules, such as neural cell adhesion molecules (N-CAM), [426] or affecting the expression of neurotransmitters and neuromodulators. [605] Interestingly, these trophic actions occur in regions of the brain that express high levels of ER mRNA, protein, and binding, as well as regions in which ER expression is low. The trophic actions appear to influence long-term potentiation and excitability, the architecture of neuronal populations, and interactions between neurons and astrocytes. Some of these developmental actions of estrogen have long-lasting and delayed "organizational" effects on behavior and hormone secretion patterns in the adult and are functionally related to sexual differentiation of the brain. Thus, many parts of the adult brain and peripheral nervous system are sexually dimorphic as a result of exposure of the brain to steroids during critical developmental periods. [521] In the adult, estrogen acts on the brain as a reproductive hormone with multiple neuroendocrine targets. It regulates the synthesis and secretory patterns of neurotransmitters and neuropeptides and their receptors that, in turn, influence sexual behavior [462] and gonadotropin and prolactin secretion. [325] These reproductive actions of estrogen have been investigated extensively. On the other hand, we are only beginning to appreciate that estrogen exerts neurotrophic and neuroprotective actions in the adult brain, as it does during development. The discovery of four types of ERs (ERα, ERβ, membrane ER, and cytosolic ER) [61, 148, 331, 495, 605] raises new possibilities for novel targets, different mechanisms of action, and differential regulation of receptor expression. Thus, the adult brain remains highly plastic and responsive to the nonreproductive effects of this hormone. This concept is supported by both clinical studies and experiments performed with laboratory animals.

1.3.2. Mechanisms of the protective effects of estrogen

It appears that estrogens exert trophic and protective actions through multiple traditional and novel mechanisms that may interact with each other under various circumstances. Because these mechanisms may interact with or impact each other, they are difficult to separate into clear and distinct categories. Nevertheless, the discussion of the potential mechanisms of action will be divided into the following two major categories: genomic actions that require significant time of exposure before trophic or protective actions are observed and that involve changes in expression of genes, and nongenomic actions that occur rapidly and do not appear to require changes in gene transcription. For the genomic actions, ERs (α and/or β) are involved as traditional estrogen-dependent transcription factors that alter transcription of target genes. The nongenomic actions appear to involve communication with one or more second messenger signaling systems and may involve novel cross-talk with ERs. In other cases, ERs may not be required for the nongenomic mechanisms of action.

1.3.2.1. Protective actions through ER-dependent genomic effects

Studies performed both *in vivo* and *in vitro* suggest that physiological concentrations of estradiol protect through ER-dependent mechanisms leading to transcription of critical genes that ultimately promote cell survival. Wise *et al.* [605] recently discovered that within 24hr of middle cerebral artery occlusion, ER messenger RNA (mRNA) is dramatically up-regulated and that estradiol pretreatment prevents injury-induced down-regulation of ER in the cerebral cortex . These data suggest that brain injury may influence responsiveness of the injured cerebral cortex to estradiol and induce differential actions that are mediated by receptor subtypes. [437] It is important to note that ER is only transiently expressed in the cerebral cortex during neonatal development when this region of the brain undergoes dramatic neurogenesis, neuritogenesis, and differentiation. Its expression virtually disappears thereafter. It has been speculated that the dramatic up-regulation of ER in the cerebral cortex may allow a recapitulation of the developmental actions of estradiol in promoting neurogenesis and redifferentiation of the cortex. Several studies support the concept that following stroke injury, specific features of brain function (*e.g.* bilateral motor control and the capacity to reorganize cortical representational maps) revert to those seen during early stages of development, with the process of recovery recapitulating ontogeny. [49, 480] Further, Dubal and Wise [156] have recently reported that physiological levels of estradiol do not protect against ischemic injury in ER knock-out mice. These data clearly establish that ER is a critical mechanistic link that mediates the neuroprotective effects of physiological levels of estradiol. Using explant cultures of the neonatal cerebral cortex, Wilson *et al.* have shown that low concentrations of estradiol protect against cell death. [603] The above studies strongly suggest that ERs are critical because the protection is achieved using 17β-estradiol (E2) and is blocked by coincubation with ICI 182,780, an ER antagonist. These findings complement those of Gollapudi and Oblinger, [212, 213] who showed that PC12 cells transfected with the full-length rat ER respond to the protective effects of estradiol, but cells transfected with vector DNA alone are not protected by estradiol. What genes are influenced by estradiol and how do these downstream events drive neuroprotection? It is well accepted that estradiol influences the expression of numerous genes in multiple regions of the brain, including the hippocampus and cortex, which are theoretically relevant to estradiol's ability to protect. For example, estradiol affects, in complex ways, the expression of genes that are involved in the balance of apoptosis and cell survival, [193, 194] mitochondrial function, [360] the function of astrocytes, [11, 85] synthesis and secretion of neurotransmitters that modulate neuronal excitability or neuron/astrocyte interactions, [392] expression of neurotrophins, growth factors, and their receptors, leading to enhanced neuronal viability, [209, 210, 397, 563] and expression of factors that influence dendritic or axonal elongation and synaptogenesis. [525] In addition, it has been reported that injury induces alterations in the expression of many of the same or functionally related genes. [2, 274, 503, 504] Thus, it is tempting to speculate that estradiol protects through modulation of these genes. However, few studies [154, 155, 157, 465] have directly tested whether estradiol influences these factors in the context of injury. In general, these studies have shown an interaction between injury and the presence of estradiol that favors the survival of neurons after injury. However, to date, no studies have established that such alterations are functional links to estradiol's ability to protect against injury-induced cell death. It has been demonstrated that estradiol's ability to protect correlates with differential expression of *galanin, bcl-2, c-fos*, and ER and ER mRNA in the cerebral cortex after ischemic injury, and various investigators are beginning to probe the functional roles of these estradiol-mediated changes in gene expression. [194, 379, 385, 465, 542]

1.3.2.2. Nonreceptor-mediated protective actions of estradiol

1.3.2.2.1. The effect of estrogen through vasculature modification

Some trophic and protective effects of estrogen which are detectable within minutes occur in the absence of action on any known intracellular ER. [39] This strongly suggests that estrogens may act either on a membrane receptor or by influencing membrane receptors that are linked to ion channels. [241, 418, 420] One example is estrogen's activity on neurotransmission which occurs by altering the activity of membrane-bound receptor systems [592] or by modulating the excitability of neuronal membranes. [241] In general, modulation of ligand-gated ion channels or G-protein-coupled receptors by such neuroactive steroids may also have consequences for nerve cell survival.

High levels of estradiol increase vasodilation and cerebral blood flow by affecting the microcirculation and vasoactive substances in the vasculature through ER-independent mechanisms. Estradiol increases cerebral perfusion in some species and under some conditions. [6] However, investigators have also reported estradiol-induced protection in the absence of changes in cerebral blood flow. [154] Therefore, it is unclear whether such vascular changes can explain the protective effects of estradiol or whether they only correlate with protection. Estrogens inhibit the vasoconstrictor endothelin and stimulate the vasodilator endothelium-derived relaxing factor, NO. [393, 394] It appears that estradiol enhances the expression and activity of two isoforms of nitric oxide synthase (NOS), endothelial NOS and neuronal NOS. [457] Pelligrino and colleagues [458, 499] reported that transient forebrain ischemia leads to a greater reduction in cerebral blood flow in ovariectomized female rats than intact females. Further, they found that this difference correlated with differences in NOS levels in the brain.

1.3.2.2.2. The effects of estrogen on free radical production

Estrogens may also protect through receptor-independent mechanisms by attenuating the formation of free radicals. At high concentrations (in the μM range *in vitro*), the phenolic A ring of estrogenic compounds acts as a highly effective electron donor and free radical scavenger, preventing lipid peroxidation-induced membrane damage. [44, 228] Several investigators [19, 313, 329, 488, 575] reported that estradiol reduces lipid peroxidation in several different neuronal cell systems and that this correlates with reduced cell death. Further, estradiol attenuates lipid peroxidation induced by various toxic stimuli, including exposure to amyloid- protein or iron sulfate. The doses of estradiol required for antioxidant activity parallel those required for neuroprotection in these systems.

1.3.2.2.3. Cross talk of estrogen with second messenger systems

Further evidence suggests that estradiol may protect against injury via receptor-dependent or receptor-independent mechanisms that involve cross-talk with other second messenger signaling molecules such as cAMP, [230, 241, 349] MAP kinases [292, 562] or molecules of the PI-3K/Akt pathway. [281] These mechanisms may allow estradiol to act rapidly through phosphorylation and activation of preexisting critical proteins and/or to act after some delay through phosphorylation-dependent genomic actions.

In summary, estradiol is rapidly recognized as a complex pleiotropic hormone that plays important nonreproductive functions in the adult brain. It is revealed that estradiol appears to act at two levels: It appears to decrease the risk of disease, and also to attenuate the extent of injury incurred by suppressing the neurotoxic stimulus itself or increasing the resilience of the brain to a given injury. Nonetheless, studies have only begun to decipher and probe the cellular and molecular basis of the novel actions of estrogen. As we continue to gain greater insights into the mechanisms of estradiol-mediated neuroprotection, we will be better able to develop estrogen-like compounds as therapeutic agents to ameliorate cognitive dysfunction and diminish the risk and severity of neurodegenerative diseases.

1.4. Calcium homeostasis and cellular function

Almost all biological responses are modulated by Ca^{++}, including muscle contraction, heartbeat and information processing, memory storage in the brain and many others. Ca^{++} acts as an intracellular messenger, relaying information within cells to regulate their activity. For example, Ca^{++} triggers life at fertilization, and controls the development and differentiation of cells into specialized types. It mediates the subsequent activity of these cells and, finally, is invariably involved in cell death. To coordinate all of these functions, Ca^{++} signals need to be flexible yet precisely regulated. This incredible versatility arises through the use of a Ca^{++}-signaling "tool kit", whereby the ion can act in the various contexts of space, time and amplitude. Different cell types then select combinations of Ca^{++} signals with the precise parameters to fit their physiology.

One of the paradoxes surrounding Ca^{++} is that it is a signal for both life and death; although elevations in Ca^{++} are necessary for it to act as a signal, prolonged increases in the concentration of Ca^{++} can be lethal. Cells avoid death either by using low-amplitude Ca^{++} signals or, more usually, by delivering the signals as brief "transients". These principles apply to both elementary and global signals. Single transients are used to activate certain cellular processes, such as secretion of cellular material in membrane-bound vesicles, or muscle contraction. However, when information has to be relayed over longer time periods, cells use repetitive signals known as Ca^{++} oscillations. Both the elementary events and the global signals can oscillate, but with widely different periods.

1.4.1. Regulation of cellular [Ca^{++}] in mammalian cells

Mammalian cells have a well programmed transport system that carefully controls access of Ca^{++} into the interior of a cell and Ca^{++} sensitive elements across the plasma membrane and redistribution of Ca^{++} from the cytosol into intracellular organelles.

1.4.1.1. Ca^{++} transport across the plasma membrane

Ca^{++} gains access into cells across the plasma membrane primarily through a number of channels, some of which are under tight control by receptors (ligand-gated Ca^{++} channels), the potential across the plasma membrane (VDCC) and the content of intracellular Ca^{++} stores (store-operated Ca^{++} channels), whereas others appear to be nonselective leak channels. [567] Ca^{++} can also gain access into the interior of cells in exchange for Na^+ by way of the plasma membrane Na^+/Ca^{++} exchanger. [529] To counteract the continuous influx of Ca^{++} into the cell, the plasma membrane contains a Ca^{++}-ATPase-type pump (PMCA) that uses ATP-dependent phosphorylation of an aspartate residue to translocate Ca^{++} from the cytosol to the extracellular environment. [95] In most tissues this pump is activated by the Ca^{++} binding protein calmodulin, which enables it to respond readily to increases in $[Ca^{++}]_i$ with an increased Ca^{++} translocating activity. Na^+/Ca^{++} exchangers also function to extrude Ca^{++} from interior of cells to extracellular spaces.

1.4.1.2. Ca^{++}-binding proteins

Once inside a cell, Ca^{++} can either interact with so-called Ca^{++}-binding proteins or become sequestered into the endoplasmic reticulum (Er), mitochondria, or nucleus. An increasingly large number of proteins have been found to have Ca^{++}-binding functions with varying binding affinities and capacities for Ca^{++}. [95, 243] Some of the intracellular binding proteins such as calmodulin act as Ca^{++} receptors. Through the Ca^{++}-protein interaction and resulting conformational change within the target protein, Ca^{++} signals can be effectively relayed and amplified. Other proteins appear to act as Ca^{++} storing devices (e.g., the calsequestrin and calreticulin families).

1.4.1.3. Intracellular Ca^{++} sequestration by organelles

The largest store of Ca^{++} in cells is found in the Er and sarcoplasmic reticula (SR), [448, 567] with local concentrations reaching millimolar levels. [567] Such high concentrations are achieved within the Er through the action of the sarco-endoplasmic reticula Ca^{++}-ATPase-type pumps referred to as SERCAs. Like their PMCA counterparts, SERCAs exist as different isoforms depending on tissue of origin, and use ATP to translocate Ca^{++} vectorially from the cytosol into the Er. It is well known that mitochondria posses a high capacity to sequester Ca^{++}. [481] Under physiologic conditions *in vivo*, total mitochondrial Ca^{++} levels and free $[Ca^{++}]$ reflect and parallel cytosolic $[Ca^{++}]$. [46] However, in a pathologic situation in which cells are exposed to high levels of Ca^{++}, mitochondria have been found to start sequestering significant amounts of Ca^{++}.[309] Mitochondria take up Ca^{++} electrophoretically through a uniport transporter. Release of Ca^{++} is accomplished by three different routes: a reversal of the uniporter, a Na^+ dependent (or independent) exchanger, and through an inner mitochondrial membrane pore that is involved in a phenomenon known as inner mitochondrial membrane permeability transition.

The transport of Ca^{++} across the nuclear membrane has been the subject of much controversy. [498] Ca^{++} must gain access to the nucleus to alter the activity of several transcription factors as part of the phenotypic effects of Ca^{++} signaling. It has been shown that a Ca^{++} wave initiated in one part of the cytosol of a cell will readily move across the nucleus. In contrast, other experiments have shown that the movement of Ca^{++} across the nuclear membrane may be restricted. Thus, despite the observation that even proteins readily permeate the nuclear membrane because of the presence of nuclear pores, the movement of Ca^{++} across the nuclear envelope has been reported to require a SERCA-like pump. [436] However, it remains unclear whether this active transport of Ca^{++} is at the level of the pores or the envelope.

1.4.2. Ca^{++} signaling in mammalian cells

The presence of intracellular Ca^{++} stores, particularly within the Er, that are in rapid equilibrium with the cytosol is the basis of the Ca^{++} signaling machinery. Mammalian cells respond to a complex array of phosphorylation events and diffusible messenger generation triggered by numerous hormones and growth factors with the production of controlled increases in $[Ca^{++}]_i$. These increases result from the combination of Ca^{++} release from intracellular stores in response to either the diffusible messenger inositol 1,4,5-trisphosphate (IP_3) or a Ca^{++} spike (Ca^{++}-induced Ca^{++} release [CICR]) and Ca^{++} influx across the plasma membrane. [139, 565] $[Ca^{++}]_i$ is often observed to rise rapidly in one particular area within a cell and then to spread rapidly as a Ca^{++} wave across the entire cell. In the continuous presence of a receptor agonist, the Ca^{++} wave is often repeated at defined intervals, producing $[Ca^{++}]_i$ oscillations. This is now viewed as a mechanism for providing the cell with a Ca^{++} signal, the frequency and amplitude encoding of which depends on the intensity and nature of the stimulus. The cell appears to benefit in several ways from this type of Ca^{++} signaling. First, the oscillating nature of the $[Ca^{++}]_i$ allows a graded response. This is best exemplified by the enzyme Ca^{++}/calmodulin kinase II, in which the kinase recruits increasing numbers of calmodulin molecules with increasing $[Ca^{++}]_i$ oscillatory frequency until the enzyme becomes fully active. A second important facet of the oscillatory nature of Ca^{++} signals is that it allows the signals to be relayed more efficiently into mitochondria [447]. Finally, it is important to remember that a prolonged elevation of $[Ca^{++}]_i$ will have detrimental effects on cell survival, leading to cell death by apoptosis or necrosis. [566] Therefore, the frequency encoding of Ca^{++} signals allows physiologic responses to occur without compromising cell survival.

The molecular basis of the Ca^{++} oscillations has been the focus of many studies. The initiator of the Ca^{++} wave is $InsP_3$, which is formed by the action of phospholipase C (PLC) on the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2); the other product of this reaction is diacylglycerol (DAG), an activator of protein kinase C (PKC) isoenzymes. Two major subfamilies of PLC exist, namely PLCβ, whose members are activated primarily by G protein coupled receptors, and PLCγ, whose members are controlled by protein tyrosine kinase receptors and protein tyrosine kinase-associated receptors. IP_3, once formed, will readily diffuse away from the site of formation to bind to Ca^{++}-release receptor channels, the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$). Three forms of $InsP_3Rs$ (types 1, 2, and 3) have been characterized by cDNA cloning. Most cells possess at least one form of $InsP_3R$ and many express all three subtypes. $InsP_3Rs$ are localized primarily on the Er as tetramers of large subunits and resemble in their structure and molecular organization the ryanodine receptors, the voltage- or Ca^{++}-sensing Ca^{++}-release channels responsible for CICR. [567] Once released into the cytosol, Ca^{++} may be recycled by re-uptake into the Er by the SERCAs, transient sequestration by mitochondria and extrusion from the cell by the

PMCAs. In fact, the latter appears predominant in cells such as hepatocytes, and this explains why Ca^{++} oscillations are short lived in the absence of extracellular Ca^{++}.[351] The refilling of Er stores therefore requires replenishing; this is achieved through a phenomenon known as store-operated Ca^{++} influx.

The essence of the system is that a sensing mechanism is present in the Er or the portion associated with the IP_3-sensitive calcium store that detects the decrease in Er Ca^{++} content caused by the discharge into the cytosol through $InsP_3R$ channel openings. This sensing mechanism then sends a signal to plasma membrane channels that are distinct from classic receptor-operated and voltage-gated channels and that are highly specific for Ca^{++}. [47, 474, 475] Whether the signal for Ca^{++} channel opening is a diffusible messenger (Ca^{++} influx factor, cGMP) or occurs through direct coupling remains controversial. An important point to note in this context is that this signal may be long lived. Therefore chemicals that inhibit SERCAs such as thapsigargin and 2,5-di (tert-butyl) hydroquinone [309] will cause a prolonged stimulation of Ca^{++} entry into cells as a result of Er Ca^{++} release. This leads to a sustained elevation of $[Ca^{++}]_i$ and thereby to an array of pathologic conditions.

1.4.3. Ca^{++} and cytotoxicity

Given the complexity of the regulation of cellular Ca^{++} and Ca^{++}-signaling processes, it is not surprising that disruption of these control mechanisms has been linked to the pathogenesis of diseases and cytotoxic events. Research has shown that a perturbation of calcium homeostasis is a common and fatal event responsible for cell death. [565, 566] It has been established that the perturbation of calcium homeostasis is caused by the inhibition of Ca^{++} transport mechanisms, including the PMCAs and

SERCAs, by cytotoxic chemicals or their metabolites. Consequently, the injured cells are exposed to a prolonged elevation of $[Ca^{++}]_i$ that in turn activates several catabolic processes catalyzed by Ca^{++}-activated proteases (calpains), phospholipases, and endonucleases. Cells undergoing apoptosis show well-defined morphologic and biochemical changes, including cellular and nuclear shrinkage, condensation, margination and fragmentation of chromatin, changes in plasma membrane architecture, and intracellular proteolysis. Ca^{++} has been found to play a pivotal role in such apoptotic cell death.

A role for Ca^{++} in initiation of apoptosis has been inferred from several observations. These include increases in $[Ca^{++}]_i$ in thymocytes undergoing apoptosis induced by glucocorticoids, [409, 597] the induction of Ca^{++}-dependent endonucleases in apoptotic thymocytes and other cells, [161] blockage of DNA fragmentation and prolongation of cell survival by treatment with Ca^{++} chelators, [328, 448] and induction of apoptosis by treatment of cells with Ca^{++} ionophores. [161] At present, Ca^{++}-induced cytotoxicity can be summarized as follows according to the various pathways utilized.

1.4.3.1. Involvement of mitochondrial dysfunction

Mitochondria are unique among cell organelles in their involvement in the concerted consumption of oxygen, production of oxygen radicals and mobilization of $[Ca^{++}]_i$. Excessive Ca^{++} accumulation in mitochondria uncouples electron transfer from ATP synthesis. Impairment of energy metabolism increases generation of free radicals. [48] Mitochondria have emerged as the missing link between elevation of $[Ca^{++}]_i$ and glutamate neurotoxicity (Fig. 1.5). [410] The critical role of mitochondria in excitotoxicity has recently been documented primarily by examining the relationship between changes in $[Ca^{++}]_i$, mitochondrial membrane potential ($\Delta\Psi$), and neuronal cell death evoked by glutamate agonists. This discussion follows the pathway outlined in Fig. 1.4. [410] Prolonged stimulation of NMDA receptors in cultured hippocampal, cortical or cerebellar granule neurons evokes a massive accumulation of Ca^{++}, [87, 88, 433] which has been estimated to reach an equivalent $[Ca^{++}]_i$ of 20μM. Persistent elevation of $[Ca^{++}]_i$, i.e. Ca^{++} overload, increases with the duration of the glutamate agonist challenge. Concomitantly, the glutamate-induced Ca^{++} overload produces neuronal death. Time-lapse fluorescence imaging of the Ca^{++} indicator Fluo-3 in hippocampal neurons identified a disproportionately higher incidence of cell death than could be accounted for by the extent of Ca^{++} overload. Such a disparity suggested that additional steps intervene between elevation of $[Ca^{++}]_i$ and cell death. Similar results were obtained by imaging cerebellar granule cells using fura-2 and forebrain neurons by indo-1 microfuorimetry [295]. These observations suggest a model in which mitochondrial homeostasis acts as the gatekeeper between Ca^{++} and cell survival and death.

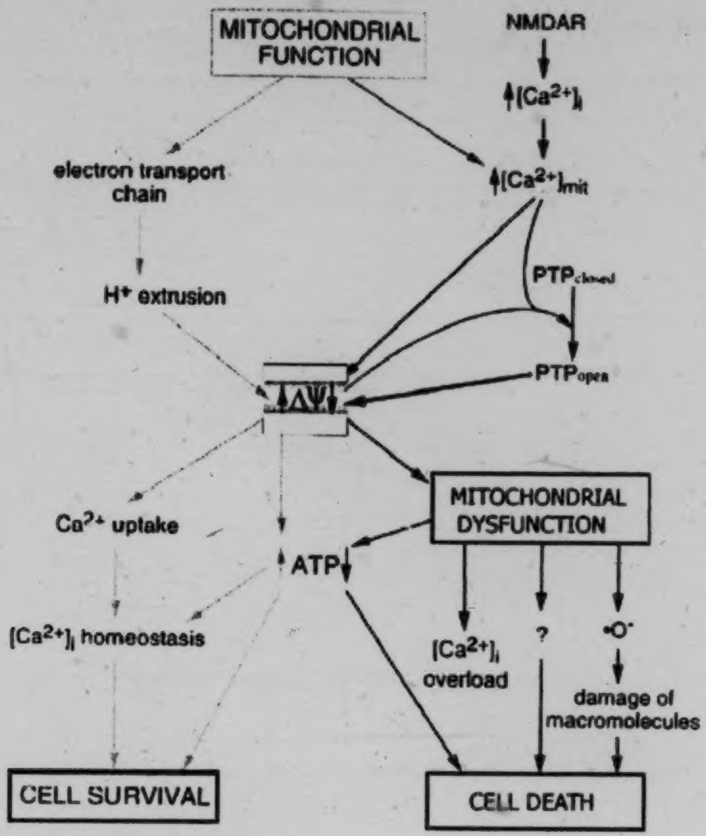

MITOCHONDRIAL FUNCTION
NMDAR
↑[Ca2+]i
↑[Ca2+]mit
electron transport chain
H+ extrusion
PTPclosed
PTPopen
↑ΔΨ↓
MITOCHONDRIAL DYSFUNCTION
Ca2+ uptake
↑ATP↓
[Ca2+]i homeostasis
[Ca2+]i overload
?
•O-
damage of macromolecules
CELL SURVIVAL
CELL DEATH

Fig. 1.5. Potential pathways by which mitochondrial dysfunction could act as an effector of excitotoxic neuronal death.

Excessive activation of the NMDAR induces massive Ca^{++} influx and abnormal elevations of [Ca^{++}]$_i$. Mitochondrial Ca^{++} uptake, driven by $\Delta\Psi$, attenuates the increases of [Ca^{++}]$_i$. This, in turn, causes a decrease in ATP synthesis and the opening of the PTP, which collapses $\Delta\Psi$. Mitochondrial dysfunction elicits a further reduction in intracellular ATP pools, increases free radical generation, and most likely activates other processes that ultimately contribute to neuronal death (modified from [410]).

1.4.3.2. Lipolysis

Phospholipids are hydrolyzed by the action of phospholipases, yielding free fatty acids and other compounds, many of which have important biological activities. It is likely that such compounds modulate the outcome of ischemia or hypoglycemic insults. [174] Inasmuch as resynthesis of the parent compounds requires energy in the form of ATP, concentrations of some of the biologically active degradation products of phospholipids represent a balance with the rate of hydrolysis, which is Ca^{++}-dependent.

Several phospholipids are broken down by PLA2 to lysophospholipids and free fatty acids, including arachidonic acid. Both types of primary degradation products can act as detergents and ionophores, thereby leading to membrane dysfunction. Both PLA2 and PLC may be activated as a result of stimulation of surface receptors. [526] Receptor coupling to PLC causes hydrolysis of PIP2 to DAG and IP3, both acting as second messengers. A major effect of DAG is to activate PKC. Such translocation is triggered by Ca^{++}. A rise in Ca^{++} can cause sustained translocation and activation of PKC, and such activation may alter the membrane by phosphorylating receptors, ion channels, or translocases. [526] Arachidonic acid produced in the pathway, working in conjunction with free radicals, has a proinflammatory effect, promoting cell death in several models.

1.4.3.3. Protein phosphorylation, gene expression, protein synthesis and calcium homeostasis

Protein synthesis, which is an energy-demanding task, is depressed when cellular ATP concentration falls, as seen in ischemia, hypoxia, and hypoglycemia. [598] During reperfusion following a transient ischemic insult, overall protein synthesis recovers very slowly or not at all, suggesting long lasting inhibition of protein synthesis. However, new mRNA transcripts are expressed in metabolically perturbed tissue, and new proteins are preferentially synthesized. New mRNA transcripts of the immediate early genes, such as *c-fos* and *c-jun,* are translated into the protein components of the AP1 complex, which by binding DNA promotor regions, triggers further transcription of other genes. [320] Synthesis of such proteins may represent a homeostatic mechanism that helps cells survive damage.

In this sequence of events, Ca^{++} probably plays a pivotal role because a rise in $[Ca^{++}]_i$ may by itself serve as a trigger for the transcription of new genes. [351] Furthermore, Ca^{++} also activates calmodulin dependent protein kinase II. As mentioned previously, these kinases may phosphorylate membrane proteins, receptors and ion channels, thereby altering membrane functions. However, together with a series of phosphatases, these and other kinases (MAP kinase and tyrosine kinases) form an intricate signal transduction chain that regulates not only gene expression, but also protein synthesis. It is possible that cell dysfunction due to a loss of cellular calcium homeostasis is mediated by sustained alteration of protein kinases and/or phosphatases.

1.4.3.4. Ca^{++} and free radicals

A link has been observed between a rise in Ca^{++} and enhanced production of free radicals. ROS-induced functional abnormalities in cardiac muscle are thought to be linked to an increase in $[Ca^{++}]_i$, which has been confirmed with the fura-2 technique. [459] The broad effects of ROS can also be explained in terms of changes in the Ca^{++} second messenger system. In cardiac tissue, the elevation of cytosolic Ca^{++} (Ca^{++} overload) is linked to various functional abnormalities, e.g., contractile dysfunction and ventricular arrhythmia, associated with ROS-induced tissue damage during ischemia reperfusion. ROS-induced changes in muscle $[Ca^{++}]_i$ homeostasis in general could be mediated via depression of sarcolemmal Ca^{++}-ATPase, inhibition of SERCA, modification of the gating of SR Ca^{++}-release channels, changes in the Na^{+}/Ca^{++} exchanger, or nonspecific Ca^{++} leakage across membranes. [423] The changes in Ca^{++} homeostasis need not be directly due to ROS-induced modifications in Ca^{++} pathways but may also arise indirectly via modifications in other ion pathways. Cai and Wise [92] have argued that H_2O_2 may modulate agonist-induced Ca^{++} influx, activating NOS, which metabolizes L-arginine to citrulline and NO, indirectly via depolarization of the membrane potential due to H_2O_2-induced inactivation of Ca^{++} activated K^{+} channels (K_{Ca} channels).

Previous research has indicated that some Ca^{++} pathways are ruled out as a cause for changes in calcium homeostasis in response to ROS. For example, the irreversible free-radical-induced decrease in Ca^{++} currents in ventricular myocytes suggests that cellular Ca^{++} overload during reperfusion is unlikely to be due to an increase in the sarcolemmal Ca^{++} influx via voltage-gated Ca^{++} channels. Regarding the contribution of other Ca^{++} pathways to changes in Ca^{++} homeostasis, Netzeband *et al.* [432] found that the Ca^{++} pump contributing to the IP_3-sensitive pool was damaged by H_2O_2 and O_2^{-}, whereas the Ca^{++} pump contributing to the IP_3-insensitive pool was only damaged by H_2O_2. The IP_3-sensitive Ca^{++} channel and a suspected RyR Ca^{++}-release channel are less sensitive to oxidative stresses than is the Ca^{++} pump.

Oxidant-induced changes in calcium homeostasis have been reported to occur in neurons. Detailed cascades of ROS-induced calcium homeostasis disruption are discussed in earlier sections concerning neurodegenerative diseases (1.1.2.3).

1.4.3.5. Cell death related to glutamate exposure and Ca^{++}

Many reports attest to the fact that transient exposure of neurons in culture to glutamate and related excitatory amino acids leads to rapid or delayed cell death and such devitalization of cells is coupled to influx of Ca^{++} from the medium. [68, 82, 87, 109, 112] There are two major controversial issues in this field. First, it has been proposed that neurons *in vitro* may be particularly susceptible to excitotoxic cell death because they lack the trophic support that cells have *in vivo*. [371] Second, it has been debated whether excitotoxicity is related to the elevation in $[Ca^{++}]_i$.

It has been suggested that, although excitotoxic damage does not the direct result of the rise in $[Ca^{++}]_i$, subsequent reports suggest that neuronal cell death correlates with the influx of Ca^{++}. [568] These investigations indicated that although both exposure to glutamate and K^+-induced depolarization increased $[Ca^{++}]_i$, only the former was toxic. They also demonstrated that independent of the $[Ca^{++}]_i$ recorded, the site of Ca^{++} influx correlates with cell death. Notably, influx of Ca^{++} through NMDAR-gated channels was potentially more detrimental than was influx through other channels. Thus, if the site of Ca^{++} entry is taken into consideration, the severity of the Ca^{++} load correlates with the damages incurred.

The major pathways of Ca^{++} dysfunction-induced cell death are outlined in Fig.1.6. In summary, Ca^{++} plays a role in several important functions in the CNS such as neurotransmitter release, neuronal plasticity, etc. However, its excessive influx to neurons due to failure of the mechanisms implicated in the regulation of its intracellular concentration (Ca^{++}-channels, calcium binding proteins) leads to a cascade of events which causes cytotoxicity and neuronal death. Ca^{++}- mediated toxicity has been implicated in the pathogenesis of neurodegenerative diseases, brain ischemia, epilepsy, cranial trauma, and the AIDS-dementia complex. Cellular calcium imbalance-induced cell death is involved in both apoptotic and necrotic neuronal death.

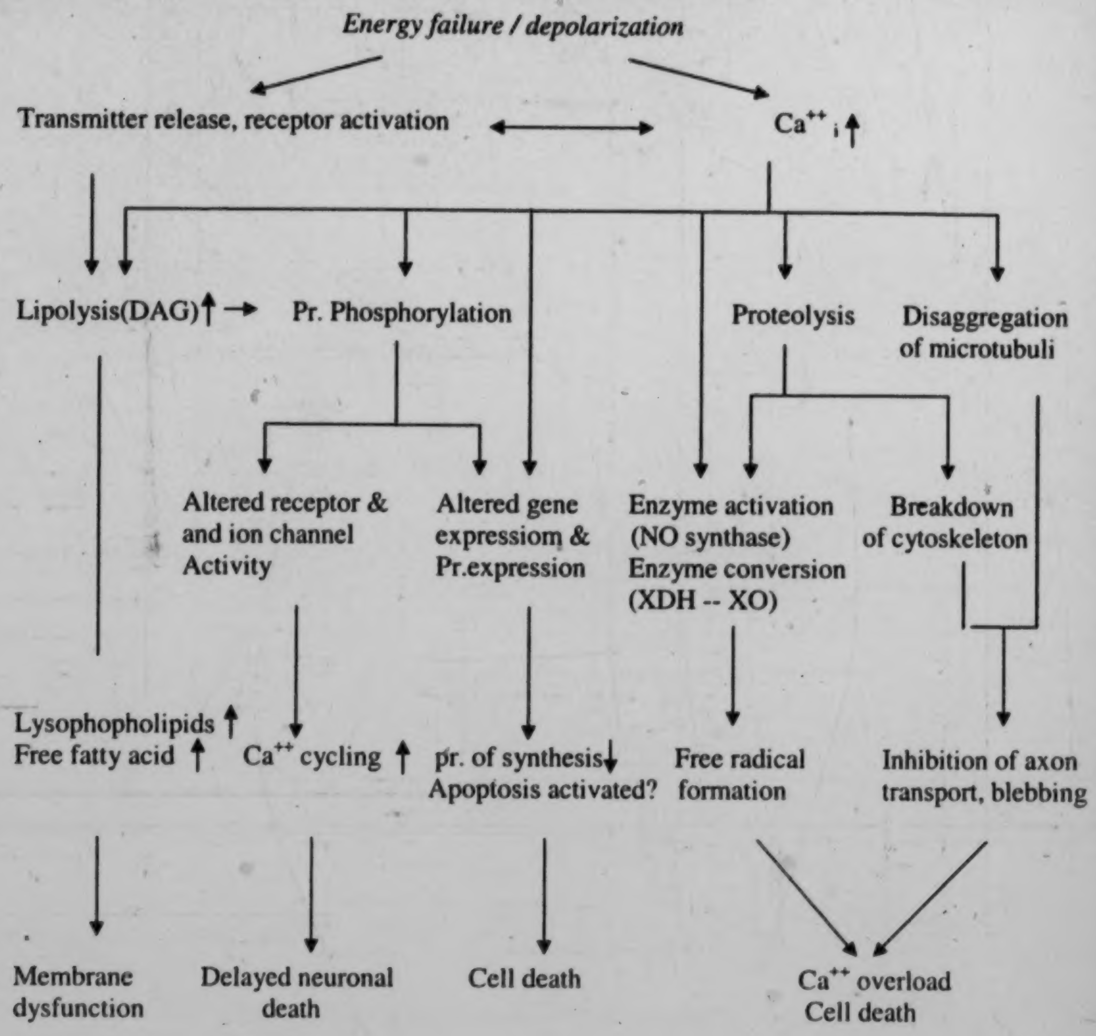

Energy failure / depolarization
Transmitter release, receptor activation
Ca++ i ↑
Lipolysis(DAG)↑
Pr. Phosphorylation
Proteolysis
Disaggregation of microtubuli
Altered receptor & and ion channel Activity
Altered gene expressiom & Pr.expression
Enzyme activation (NO synthase) Enzyme conversion (XDH -- XO)
Breakdown of cytoskeleton
Lysophopholipids ↑ Free fatty acid ↑
Ca++ cycling ↑
pr. of synthesis↓ Apoptosis activated?
Free radical formation
Inhibition of axon transport, blebbing
Membrane dysfunction
Delayed neuronal death
Cell death
Ca++ overload Cell death

Fig. 1.6. Major pathways of Ca^{++} dysfunction-induced cell death.

Diagram illustrating the effects of depolarization/receptor activation and the secondary effects of increased $[Ca^{++}]_i$ (adapted from [526]).

1.5. Overall Hypotheses

The overall hypotheses of the current study are:

1). Estrogen acts as a neuroprotectant and can protect neurons from cell death induced by different insults;

2). The neuroprotection exerted by estrogen is through multiple anti-apoptotic and anti-necrotic pathways;

3). The mechanism of the protective effects of estrogen is at least partially through stabilizing cellular calcium homeostasis.

Based on these hypotheses, the present studies have been conducted to investigate the cellular mechanisms involved in the neuroprotective effects of estrogen, and to determine how estrogen modulates calcium homeostasis.

Experiments were carried out as follows:

1). Characterization of different cytotoxic models;

2). Testing the anti-cytotoxicity effects of estrogen in SK-N-SH cell culture;

3). Investigation of the effect of estrogen on cellular metabolism.

1.6. Specific Aims

1). To establish cell culture models of cytotoxicity to study potential neuroprotective agents;

2). To investigate the neuroprotective effects of estrogen with the above systems, and to illustrate whether the protective effects of estrogen are through anti-apoptotic pathways;

3). To identify whether the effects of estrogen are ER-dependent;

4). To examine how estrogen modulates cellular calcium homeostasis. The effects of estrogen on VDCC activity, Ca^{++} influx, and calcium channel protein expression will be studied.

Chapter 2

Materials and Methods

2.1. Cell culture

SK-N-SH cells (passage 31) were obtained from ATCC (Rockville, MD, USA). They were routinely cultured in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 2mM L-glutamine and MEM non-essential amino acids (L-alanine.HCl, 8.9, L-asparagine.H_2O, 15, L-aspartic acid, 13.3, L-glutamic acid, 14.7, glycine, 7.5, L-proline, 11.5, and L-serine, 10.5mg/L) (Life Technologies Canada, Burlington, ON, Canada). Cells were cultured in monolayer in 100 x 20 mm^2 plastic dishes (Life Technologies Canada, Burlington, ON, Canada) at 37°C and under 5% CO_2 and 95% air. Medium was changed every 3 days, and the cells were passed once they reached about 80% confluence. Cells were not used after being passed 10 times. Cells were observed with a Nikon TMS phase contrast microscope (Nikon Canada Co., Toronto, ON, Canada) and cell counting was performed with a hemocytometer (Fisher Scientific Canada, Richmond, BC, Canada).

To initiate the experiment, culture medium was decanted, and cells were covered with 3ml of 0.05% typsin-0.53mM ethylenediaminetetraacetic acid (EDTA)-$4Na^+$ (Life Technologies Canada, Burlington, ON, Canada), and incubated at room temperature (20 ~22°C) until most cells were rounded up. After the trypsin-EDTA solution was aspirated, 10ml of fresh phenol red-free MEM (Life Technologies Canada, Burlington, ON, Canada) was added immediately. Cells were dissociated by mild trituration with a fire-polished Pasteur pipette, then collected into a 50ml centrifuge tube (Life Technologies Canada, Burlington, ON, Canada), and centrifuged at 300xg for 7 minutes. Cells were resuspended in the appropriate treatment media in 96 well flat bottom Falcon plates (Life Technologies Canada, Burlington, ON, Canada) with 100µl medium per well. Phenol red-free medium was used for all cell viability studies with WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate).

2.2 Cell viability:

Cell number was determined using the cell proliferation reagent WST-1 assay [296] (F. Hoffmann-La Roche Ltd, Postfach, Switzerland) which is a colorimetric assay for the quantification of cell proliferation and cell viability. WST-1 is a tetrazolium salt, and it is cleaved to formazan by dehydrogenases. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture. The formazan dye produced by metabolically active cells can be quantified with a scanning multiwell spectrophotometer by measuring the absorbance of the dye solution at appropriate wavelengths. To quantify cell viability, 10μl WST-1 reagent was added to each well, and the incubation continued for 4hr in the incubator (37°C, 5% CO_2). The appropriate incubation time after the addition of WST-1 was chosen by comparing the measurements of the absorption repeatedly at 0.5, 1, 2 and 4hr after the addition of WST-1 in a preliminary experiment. After the incubation, the culture plates were rocked thoroughly for 1 minute at 250rpm/min on a shaker. The absorbance of the samples was then measured against the background control, in which the same volume of culture medium and WST-1 was used as the blank, using a microtiter plate (ELISA) reader (MTX Labsystems, Helsinki, Finland). The wavelength for measuring the absorbance was 450nm, and a reference wavelength of 650nm was chosen. A standard was set up every time prior to the addition of WST-1 by preparing a serial dilution of known cell densities. Cell densities were determined by using the trypan blue dye exclusion method with a hemocytometer. Preliminary experiments comparing the accuracy of the WST-1 method with the traditional cell count method showed that the cell viability as detected with WST-1 paralleled very well with the cell number measured using the trypan blue exclusion method.

2.3. Cytotoxicity models

Four cytotoxic models were used in the current project. They were Aβ, MPTP, serum deprivation and high density induced cell death. Cells were cultured in phenol red-free MEM, and reagents were added when experiments were initiated.

2.3.1. Aβ model

Lyophilized Aβ (peptide fragment 25~35) was dissolved in reverse osmosis water (ROH$_2$O) at a concentration of 1mM, and was then diluted in phenol red-free MEM to a final concentration of 20μM. Cells were plated in triplicate at densities ranging from about 5 x 10^2 to 10^5 cells per well with a total volume of 100μl/well in a 96 well plate, and the density of 3 x 10^3 cells/well was found to be optimal. Cell viability was assessed after 48, 72, or 96hr of culture.

2.3.2. MPTP model

MPTP was freshly prepared in phenol red-free MEM 30 minutes prior to the start of experiments at 10mM concentration, and was then diluted in phenol red-free MEM to a final concentration of 0.5, 0.75 or 1mM. Experiments were performed in the same way as in the Aβ model.

2.3.3. Serum-deprivation model

SK-N-SH cells were cultured in serum-free, or low serum (1% ~ 5% FBS) medium for 48, 72, or 96hr, and then assayed for cell viability. The same cell densities were used as in the Aβ and MPTP models.

2.3.4. High density culture model

Cells were plated at a density of 2×10^4 cells/well in phenol red-free MEM, and the same culture and assay conditions were applied as in the other models.

2.4. Detection of apoptosis in cell culture

Cell death can be defined morphologically and biochemically into two distinct pathways, necrosis and apoptosis. To characterize the type of cell death, two methods were used to identify apoptosis; ELISA quantification of histone-associated DNA fragments and apoptotic DNA ladder detection.

2.4.1. ELISA quantification of histone-associated mono- or oligonucleosomes

The cell death detection ELISA method (F. Hoffmann-La Roche Ltd, Postfach, Switzerland) is a photometric enzyme-immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after apoptotic cell death occurs.

2.4.1.1.Principle of the assay

The assay is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones [74]. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. The sample (cell-lysate) is placed into a streptavidin-coated microtiter plate (MTP). A mixture of anti-histone-biotin and anti-DNA-peroxidase (POD) are added and incubated. During the incubation period, the anti-histone antibody binds to the histone-component of the nucleosomes and this immunocomplex binds to the streptavidin-coated MTP via its biotinmoiety. Additionally, the anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes. A washing step removes unbound antibodies. The amount of nucleosomes can be quantitatively determined by the POD retained in the immunocomplex, and the bound POD is determined photometrically with 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) as substrate.

2.4.1.2. Experimental procedure

Two sets of cell cultures were set up in 96 well plates in the same manner as in the cell viability study with all four cytotoxic models. After 96hr culture, one set of plates was assayed for cell viability; the second set of plates was analyzed for apoptosis starting with centrifugation of the plate at 200xg for 10 min. The supernatant was removed with care, and the cell pellet was resuspended in 200μl lysing buffer supplied with the kit. The cells were then lysed for 30min at room temperature, and then by centrifugation at 200xg for 10 min. To analyze, 20μl of the supernatant which was the cytoplasmic fraction from each well was then transferred to the stretavidin-coated MTP. Positive control (supplied with the kit), negative control (culture supernatant) and background control (incubation buffer within the kit) were also pipetted into the precoated MTP. A total volume of 80µl of the immunoreagent which consisted of incubation buffer, anti-histone-biotin and anti-DNA-POD was then added to each well. The plate was incubated on a shaker with gentle shaking (300 rpm) for 2hr at room temperature. The solution was removed by suction after incubation, and each well was rinsed 3 times with 250-300µl of the incubation buffer. The washing solution was then removed carefully, and 100 µl ABTS solution was pipetted into each well. The plate was incubated on a plate shaker again at 250 rpm until the color development was sufficient for a photometric analysis (after approximately 10-20 min.). The plate was read at 405 nm against ABTS solution as a blank. A reference wavelength was set at 490 nm.

2.4.1.3. Analysis of the results

The values were averaged from the double absorbance measurements of the samples, and the background values of the immunoassay were subtracted from each of these averages. A specific enrichment factor of the mono- and oligonucleosomes released into the cytoplasm was calculated from these values using the following formula:

$$\begin{array}{c}\text{enrichment factor}\\ \text{(the capability of inducing apoptosis)}\end{array} = \frac{\text{OD(405)} - \text{OD(490)}}{\text{cell number}}$$

OD= optimal density, and OD(405) correlates with the quantitative determination of the amount of bound fragmented nucleosomes by the POD retained in the immunocomplex photometrically using ABTS as substrate. Therefore, OD(405) proportionally represents severity of apoptosis in the culture.

2.4.2. Apoptotic DNA ladder detection

The Apoptotic DNA Ladder Kit (F. Hoffmann-La Roche Ltd, Postfach, Switzerland) is designed for the purification of nucleic acids from different sample materials like whole blood and cultured cells to detect the typical DNA ladder, [577] the hallmark of apoptotic cells. [450]

2.4.2.1. Principle of the test

The endogenous endonuclease cleaves double stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes when apoptosis occurs. The DNA fragments yielded in apoptotic cell death are discrete multiples of an 180 base pair (bp) subunit which is detected as a "DNA-ladder" on agarose gels after extraction and separation of the fragmented DNA. The cleavage of genomic DNA into nucleosome-size fragments is considered a specific hallmark of apoptosis. With the current method, after lysis of cultured cells in the binding buffer, the lysate is applied to a filter tube with glass fiber fleece and filtered through by centrifugation. During this procedure, nucleic acids bind specifically to the surface of glass fibers in the presence of chaotropic salts. Residual impurities are removed by a wash step and subsequently DNA is eluted in elution buffer. [576]

2.4.2.2. Experimental procedure2.4.2.2.1. Isolation of DNA from cultured cells

For the isolation of DNA from cultured cells, 2 x 10^6 cells were used, and 200µl PBS was added to the sample material for resuspension of the cells. Next, 200 μl of the binding buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100, pH 4.4) was added to each group to a final volume of 400μl, and the vial was mixed immediately by vortexing. Samples were incubated for 10 min at 15-25°C. For the positive control (lyophilized apoptotic U937 cells) supplied with the kit, 400μl of binding/lysing buffer was added to the vial and then followed by a 10 min incubation at room temperature. After the 10 min incubation, 100 μl of isopropanol was added to samples and the positive control, and the vials were shaken on a vortex. A filter tube and a collection tube were assembled, and the samples or the positive control were pipetted into the upper reservoir. The tubes were centrifuged for 1 min at 8000 rpm in a standard table top centrifuge (Fisher Scientific Canada, Richmond, BC, Canada) for washing. The flowthrough was then discarded. The wash was repeated 3 times, and the final 10 sec of the centrifugation for the last washing was at maximal speed (13, 000 rpm) to remove residual wash buffer. Prewarmed (70°C) elution buffer (10 mM Tris, pH 8.5) with a volume of 200μl was added to each filter tube, and the tubes were centrifuged for 1 min at 8000 rpm to elute bound DNA.

DNA is stable and can be used directly or stored at -20 °C for later analysis.

2.4.2.2.2. DNA gel electrophrosis

DNA gel electrophoresis was performed on a Hoefer HE 33 mini submarine electrophoresis unit (Amersham Pharmacia Biotech, Buckinghamshire, UK). A 1% DNA-agarose gel was prepared by adding in an Erlenmeyer flask 1g agarose (Bio-Rad Laboratories, Berkley, CA, USA) to 100 ml TBE-buffer which consisted of 44.5mM Tris (Fisher Scientific Canada, Richmond, BC, Canada), 45mM boric acid, and 1mM EDTA (pH 8.0 ± 0.1). The flask was then microwaved at maximum energy level until the agarose was completely dissolved. When the solution cooled down to 60°C, 5 μl ethidium bromide (Bio-Rad Laboratories, Berkley, CA, USA) stock solution (50 mg ethidium bromide in 5 ml ROH_2O) was added. The gel was then poured into the prepared electrophoresis chamber to a depth of 7mm, and the gel comb was placed. Once the gel was hardened, the comb was removed. Before loading the samples, the positive control and each DNA sample were mixed with loading buffer at the ratio of 4:1 in volume. A 10x loading buffer containing 1% sodium dodecyl sulfate (SDS), 0.25% bromophenol blue, and 30% glycerol, was prepared in advance. Sufficient TBE buffer was added to cover the gel to a depth of about 1 mm. Samples were loaded carefully into the sample pockets using a micropipette. The gel was run for approximately 1.5hr at 75V (Bio-Rad 3000xi computer controlled eletrophoresis power supply, Bio-Rad Laboratories, Berkley, CA, USA). When the bromophenol blue dye from the loading buffer had migrated to the end of the gel, the run was stopped, the DNA was visualized by placing the gel onto an UV light source. The gel was photographed to provide a record.

2.5. Intracellular calcium $[Ca^{++}]_i$ Determination

Quantitative measurement of $[Ca^{++}]_i$ is a critical evaluation of calcium homeostasis related to many biological responses. $[Ca^{++}]_i$ was determined according to the method previously described by Grynkiewic *et al.* [239] with modifications. $[Ca^{++}]_i$ was determined in SK-N-SH cells, or GH3 cells for comparison studies, after cells were challenged by 30mM KCl.

2.5.1. Principle of the assay

Fura-2 is a UV-light excitable, ratiometric Ca^{++} indicator which is used in most ratio-imaging experiments, in which it is more practical to change excitation wavelengths than emission wavelengths. Upon binding to Ca^{++}, fura-2 exhibits an absorption shift that can be observed by scanning the excitation spectrum between 300 and 400nm, while monitoring the emission at ~510nm. The acetoxymethyl (AM) ester of fura-2 can diffuse passively across cell membranes. Once inside the cell, the ester is cleaved by intracellular esterases to yield cell-impermeant fura-2.

2.5.2. Procedure of experiments

SK-N-SH cells or GH3 cells for positive control were cultured on 1.0 x 2.2cm^2 coverslips (Fisher Scientific Canada, Richmond, BC, Canada) at the density of 10,000 cells/coverslip in 35mm cell culture plates (Corning, Life Technologies Canada, Burlington, ON, Canada) for all treatment groups. After 96hr, cells were about 80% confluent on the coverslips, the old culture media were removed, and replaced with fresh media. Fura-2 AM (Molecular Probes, Eugene, OR, USA) dissolved in DMSO as 1mM solution was added to each well at a final concentration of 5μM, and the loading was carried out in a dark environment for 50min (a period allowing for the de-esterification reaction of the cell permeant AM ester of fura-2, and maximal binding to $Ca^{++}{}_i$) at room temperature. The cells were then washed and equilibrated in the 5K recording buffer containing (in mM) NaCl 145, KCl 5, $MgCl_2$ 2, D-glucose 10, NaH_2PO_4 0.5, HEPES 10, and $CaCl_2$ 2, pH 7.4. The coverslips were mounted in a quartz cuvette and perfused at 1.5ml/min alternately with low potassium 5K solution or high potassium 30K solution containing 30mM KCl. Using KCl as a stimulator, the intensity ratio and $[Ca^{++}]_i$ were recorded with a Photon Technologies International RCR fluorimeter. $[Ca^{++}]_i$ was calculated using the following equation, $[Ca^{++}]_i$ (nm) $= Kd \times (R-R_{min})/(R_{max}-R) \times b$ where R was the fluorescence ratio of fura-2 excited at 340nm and 380nm. The emission wavelength was set at 510nM. $F_{\lambda 1}$ (340nM) and $F_{\lambda 2}$ (380nM) are the absorbance under the respective fluorescent excitation wavelengths for the ion-bound and ion-free indicator. R_{max} and R_{min} were the fluorescence ratio determined by adding 2μM ionomycin (Calbiochem, La Jolla, CA, USA) and/or 0.5% triton, and subsequently adding 5mM EGTA, respectively. b was the ratio of fluorescent of fura-2 when excited at 380nm in the zero and saturating Ca^{++}. K_d was the dissociation constance of fura-2 for Ca^{++}, assumed to be 224nm.

2.6. Ca^{++} channel study by patch clamp

Ca^{++} channel current recordings were obtained using the whole cell version of the patch-clamp technique. [250] Patch electrodes were pulled from borosilicate glass capillary tubes (od, 1.2 mm; id, 0.9 mm; FHC, Brunswick, ME, USA) and heat polished. They were filled with a solution containing 70 mM Cs_2-aspartate, 20 mM HEPES, 11 mM EGTA, 1 mM $CaCl_2$, 5 mM $MgCl_2 \cdot 6H_2O$, 5 mM glucose, 5 mM ATP-Na_2, and 5 mM K-succinate. Creatine phosphokinase (50 U/ml) and phosphocreatine-Na_2 (20 mM) were added to the pipette solution to reduce current run down. The extracellular bath solution contained 105 mM Tris-Cl, 0.8 mM $MgCl_2 \cdot 6H_2O$, 5.4 mM KCl, 20 mM $BaCl_2$, 0.02 mM tetrodotoxin, and 10 mM HEPES. Ba^{++} (20 mM) was used as the charge carrier. All solutions were filtered (0.22 μm) before use. The osmolarity was adjusted to 320 mosmol, and the pH was adjusted to 7.4. The membrane currents were measured using an Axopatch 1B whole cell patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). The data were filtered at 5 kHz and sampled at 10 kHz using pClamp software (pClamp 7) and a Digidata 1200B analog to digital interface (Axon Instruments, Foster City, CA, USA). Analysis was performed using the pClamp software. To generate current-voltage (I-V) relationships, 250-msec depolarizing test pulses of increasing amplitude were applied at a frequency of 0.3 Hz. On-line leakage subtraction was implemented using the P/2 protocol in pClamp software. At a holding potential of -50 mV and with Cs^+ in the internal solution, hyperpolarizing pulses did not activate any currents, and identical results were obtained with the P/2 or P/4 protocol. The experiments were performed at room temperature.

2.7. Western blotting

2.7.1. Principle of the methodology

Western blotting is used to identify specific antigens recognized by polyclonal or monoclonal antibodies [222]. Protein samples are solubilized, usually with sodium dodecyl sulfate (SDS) and the reducing agent dithiothreitol (DTT). Following solubilization, the material is separated by SDS-page gel electrophoresis. The antigens are then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The transferred proteins are bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining binding sites are blocked by immersing the membrane in a solution containing blocking agents. After probing with the primary antibody, the antibody-antigen complexes are identified with horseradish peroxidase (HRP) coupled to the secondary antibody. Luminescent substrates are then used to visualize the activity. As indicated, the HRP/hydrogen peroxide-catalysed oxidation of luminol in alkaline conditions is the principle of the detection system chosen in our experiments. Immediately following oxidation, the luminol is in an excited state which then decays to the ground state via a light emitting pathway. [491] The maximal light emission is at a wavelength of 428nm, which can be detected by a short exposure to blue light-sensitive autoradiography film.

2.7.2. Experimental procedure

2.7.2.1. Sample protein preparation

SK-N-SH cells were cultured in 6-well cell culture plates (Corning, Life Technologies Canada, Berlington, ON, Canada) for 96hr, and were then harvested by centrifugation at 200xg for 7min after being washed twice in Hank's balanced salt solution (HBSS) containing protease inhibitor cocktail solution. The protease inhibitor cocktail consisted of pepstatin A (1 μg/ml), leupeptin (1 μg/ml), aprotinin (1 μg/ml), Pefabloc SC (0.2 mM), benzamidine (0.1mg/ml), and calpain inhibitors I and II (8 μg/ml each). An aliquot of the cells was resuspended in ROH_2O in a 1.5ml microtube, vortexed and ground with a Kontes Pellet Pestle (Fisher Scientific Canada, Richmond, BC, Canada), and then assayed for protein concentration with the BCA protein assay (Pierce, Rockford, IL, USA). The rest of the cells were resuspended in reducing sample buffer (0.5M Tris-HCl 25%, SDS 4%, Glycerol 20%, Bromophenol blue 0.01%, and 50mM DTT), vortexed and ground, and were boiled at 100°C for 5min.

2.7.2.2. Dot blotting

To determine the optimal titers of the antibodies, dot blotting was applied to all primary antibodies. The PVDF membrane (Millipore, Bedford, MA, USA) was soaked in methanol for 10sec, then washed twice with ROH_2O, and equilibrated in transferring buffer containing 25mM Tris, 200mM glycine and 20% methanol for 15 min. Protein samples with a total protein content of 10μg were added to the premarked dots on the membrane, and the membrane was airdried for 2h. The membrane was subsequently washed 3 times in TBST buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween-20). This was followed by blocking incubation with 5% non-fat milk and 1% bovine serum albumin (BSA) for 1h. The membrane was washed, and primary antibody applied at different titers, and the membrane was incubated in a humid chamber for 1h at 4°C. The membrane was washed 3 times with TBST when the incubation was completed. The HRP-conjugated secondary antibody was applied after the washing, and incubated for 1h at room temperature. Finally, the membranes were washed and exposed to enhanced chemiluminescence (ECL), (Amersham Pharmacia Biotech, Buckinghamshire, UK). They were then exposed to X-ray films and developed to visualize the signals. An optimal titer of the antibody was determined for each antibody according to the densitometry of the blot, and the optimal titers for anti-ERα monoclonal antibody, anti-ERβ polyclonal antibody, polyclonal anti-L-type Ca^{++} channel α_{1C} subunit (anti-α_{1C}), polyclonal anti-L-type Ca^{++} channel α_{1D} subunit (anti-α_{1D}), and monoclonal anti-dihydropyridine (DHP) binding complex α_1-subunit (anti-α_1), were 1:2000, 1:1000, 1:1000, 1:600, and 1:600, respectively.

2.7.2.3. Electrophoretic separation of protein

Gel eletrophrosis under denaturing conditions separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode. Prior to electrophoresis, 10 ~ 40μg of protein were loaded per lane on 7% SDS-polyacrylamide minigels. The first well of the gel was always loaded with 5μl of the prestained Kaleidoscope protein standard (Bio-Rad Laboratories, Berkley, CA, USA). The gels were run at 75V (Bio-Rad 3000xi computer controlled electrophoresis power supply) until the bromphenol blue tracking dye entered the separating gel, and the voltage was then increased to 140V. The run was stopped when the 124Kd band of the standard ran into the lower 1/3 of the gel.

2.7.2.4. Transfer and blot

When electrophoresis was complete, the gel was equilibrated for 15min at room temperature in transferring buffer. PDVF membrane was prepared in the same way as in the dot blotting procedure. The proteins on the gel were transferred to the membrane for 1h 30min at 75v, 150mA with cooling in a cold room. The membrane was removed from the apparatus, and immunoprobing proceeded with visual detection of proteins using the same operation as in the dot blotting.

2.7.2.5. The proteins identified in SK-N-SH cells with the Western blot technique

To identify the expression of ERs in SK-N-SH cells in the study of the neuroprotective effects of E2, Western blotting with anti-ERα monoclonal antibody and anti-ERβ polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was performed. Additionally, L-type VDCC expression was investigated by identifying the α_1 subunits of the L-type VDCCs. The expression of α_1 (DHP binding complex), and 2 isoforms of neuronal α_1 subunits, α_{1C} and α_{1D} were tested by using Western blot analysis with monoclonal anti-α_1 (BioMol Research Laboratories Inc., Plymouth Meeting, PA, USA), polyclonal anti-α_{1C}, and polyclonal anti-α_{1D} (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), respectively.

2.7.2.6. Densitometry analysis of the blotting

Un-Scan-It gel 5.1 software (Silk Scientific, Orem, UT, USA) was used to analyze the density of the bands to compare protein expression under different conditions.

2.8. Statistical analysis

All data are presented as means ± SEM. The significance of differences was determined by Student's t-test for 2 group comparison, or one-way ANOVA with Newman-Keuls as a *post hoc* test for multiple group comparisons with GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA, USA). $P<0.05$ was considered as significant difference in all experiments.

*All reagents from Sigma Chemical Corporation, St. Louis, MO, USA unless otherwise indicated.

Chapter 3

Cytotoxic Models with SK-N-SH Cell Culture

3.1. Introduction

Cytotoxicity is simply the cell-killing property of a chemical or a mediator cell, independent from the mechanisms of cell death. The purpose of setting up a reliable cytotoxic model is to test the protective effects of potential neuroprotectants. The cytotoxic models established in the current studies are Aβ, MPTP, high density culture and serum deprivation-induced neuronal death with SK-N-SH cell culture.

3.1.1. Aβ cytotoxicity model

Aβ, a 4 kDa protein of 39~43 amino acids, is a metabolic product of the membrane spanning, 695~770 amino acid precursor protein, βAPP. Aβ deposit is one hallmark of AD. The change of conformation and fibrillar aggregation of Aβ result in extracellular deposits, which interact with cells and may lead to neuronal death. This has been confirmed by many studies using cerebral cells in primary culture or neuronal cell lines. [43, 93, 264, 279, 383] The toxic portion of the Aβ molecule appears to be the 25~35 fragment [378] which is used in the current study. The Aβ 25~35 fragment causes cell death in primary neuronal cultures with a dose-response and time-course similar to that observed with Aβ 1~40. Additionally, Aβ 25~35 tends to form aggregation in aqueous solutions more efficiently. [218, 378] It has been shown that Aβ neurotoxicity is manifested by its aggregative form. [388] The pathway by which Aβ induces neuronal cell death has been investigated and various mechanisms have been proposed, including $[Ca^{++}]_i$ accumulation, NO and peroxide production, membrane lipid peroxidation, decreased membrane fluidity, and alteration of the cytoskeleton and nucleus. All of these intracellular events converge to the ubiquitous pathways of necrosis or apoptosis. The presence of Aβ in neuronal cultures may cause a sustained binding of the molecule to putative cell surfaces and/or intracellular receptors and activate signal transduction cascades which compromise a broad range of intracellular mechanisms essential to the maintenance of neural functioning. Two major molecular cascades, excitotoxicity and free radical induced oxidative stress, have been implicated in Aβ cytotoxicity. [256] In neuronal cell lines, Aβ toxicity induces the production of ROS and NO. The production of these compounds is accomplished by depletion in glutathione peroxidase (GSH), activation of inducible NO synthase (iNOS), accumulation of mitochondrial peroxynitrite ($ONOO^-$), tyrosine nitration, and membrane lipid peroxidation. *Bcl-2* expression, soluble amyloid precursor protein (sAPP), TNF-α and C2-ceramide pretreatment protect neurons against Aβ-induced oxidative stress associated with increased expression of GSH and manganese superoxide dismutase (Mn-SOD). [84, 314] A substantial cytosolic increase in calcium can induce cell death. Data on the calcium flux involved in Aβ-induced toxicity are contradictory. Two phases of the toxic process have been described upon Aβ exposure: an early Ca^{++}-independent phase and a late Ca^{++}-dependent phase involving the VDCCs, such as the L-type VDCC, and intracellular flux from the endoplasmic reticulum through the IP_3 receptor or ryanodine receptor. [104] In some studies, no variation in $[Ca^{++}]_i$ leading to Aβ toxicity has been observed. [192]

Although the data remain controversial, evidence is accumulating for two types of cell death in AD: apoptosis and necrosis. Necrosis is reported to be induced by Aβ in PC12 cells, [38] and by an inflammatory response of glial cells. Morphological changes, characteristics of apoptosis (membrane blebbing, nuclear chromatin condensation, DNA fragmentation), and modulation of expression of apoptotic genes (decreased expression of *Bcl-2* and increased expression of *Bax*, *c-fos*, *c-jun*, p53, and Fas) are all induced by Aβ in neuronal primary culture and AD postmortem tissue. [134, 171, 540] However, determining whether Aβ directly triggers neuronal cell death or whether its effects are relayed by activated microglia requires further investigation.

3.1.2. MPTP cytotoxicity

The neurotoxin MPTP destroys the dopaminergic neurons in the SN of humans and monkeys, and causes Parkinsonism in these affected individuals. [303, 338, 570] MPTP has a complex multistep metabolism. It is a highly lipophilic compound. Thus, after its systemic administration, it can quickly gain access to the brain by freely crossing the blood-brain barrier. [183, 255] Once in the brain, MPTP is converted into its active metabolite, MPP^+, by MAO-B within nondopaminergic neurons (for example, serotonergic neurons and glial cells). [205, 280, 611] The next important step in the MPTP neurotoxic pathway is the mandatory entry of MPP^+ into dopaminergic neurons. Because MPP^+ is a polar molecule, in contrast to its precursor MPTP, it can not freely enter into cells, but depends on plasma membrane carriers. MPP^+ has a high affinity for the plasma membrane dopamine transporter (DAT) as well as for norepinephrine and serotonin transporters. The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists (for example, mazindol) completely prevents MPTP-induced toxicity. [574] Intracellular MPP^+ can also be taken up and concentrated within the mitochondria where it blocks complex I of the electron transport chain, which in turn decreases the production of ATP and increases the formation of free radicals such as superoxide. [147, 514] Although the actual impact of MPP^+ -induced ATP deficit in MPTP neurotoxicity remains to be demonstrated in living animals, available data have supported the critical role played by free radical production in the MPTP toxic process. This issue was assessed by testing MPTP toxicity in transgenic mice with increased activity of superoxide dismutase (SOD), the key enzyme in the detoxification of superoxide. These mice have 2.5-3 times greater brain SOD activity compared with wild-type littermates. Thus, MPTP administration caused significant damage in the wild-type animals, but in contrast, no significant damage was detected in the transgenic animals with increased SOD. These data indicate that superoxide plays a role in the molecular mechanism of MPTP *in vivo*. However, superoxide is probably better known as an intermediate species in various cellular reactions rather than being deleterious *per se*. [236] In contrast, superoxide can react with NO to produce highly reactive and deleterious species such as peroxynitrite. Consistent with the idea that part of the MPTP neurotoxic process involves the reaction between superoxide and NO is a demonstration that inhibition of NOS protects, in a dose dependent fashion, against MPTP toxicity. [237, 473]

Multiple lines of evidence have shown that MPTP induces apoptotic neuronal death in primary cultures and cell lines. [153, 214, 361, 472, 523] There is one report indicating that a high concentration MPTP induces necrosis, while a low concentration induces apoptosis in a rat embryo mesencephalon primary culture. [184]

3.1.3. Cell death induced by serum deprivation

Serum deprivation is a well established model in neuronal apoptosis. It induces apoptosis through the Ras signaling/Jun kinase pathway or by activation of pro-apoptotic proteins when growth factors in serum are withdrawn, and it has been recognized in PC12 cells, a murine neuroblastoma cell line N18, and some primary culture models. [17, [72, 89, 172, 467] For instance, Liu and Zhu [632] observed that serum deprivation-induced apoptosis was accompanied by a rapid down-regulation of p53, *Bcl-2* and an up-regulation of c-Myc, *Bax* and caspase-3 activity. Pretreatment with basic fibroblast growth factor (bFGF) prevented the apoptosis with an attenuation of the changes of p53, *Bcl-2*, *Bax* levels and caspase-3 activity but had no effect on the changes in c-Myc levels. These results suggest that serum deprivation induces apoptosis through a signaling pathway involving p53, *Bcl-2, Bax*, c-Myc and caspase-3. Since glutamate down-regulates *Bcl-2,* the anti-apoptotic potential of bFGF is probably due to its effects on attenuating glutamate action, and subsequently blocking the apoptosis cascade. [355] Other results showed that within 24hr of serum deprivation, apoptosis occurred in up to 65-70% of PC12 cells, and significant levels of NO were generated. Addition of TNF-α in serum-free medium increased the levels of iNOS and apoptosis compared with those in serum-deprived cells. The same group has demonstrated that differential generation of NO levels by TNF-α under conditions of serum deprivation is mediated by the same pattern of iNOS induction. NO formation via iNOS induction resulted in the activation of *c-jun* N-terminal kinase (JNK). That study suggested that the differential formation of cytotoxic NO by serum deprivation plus TNF-α is primarily mediated by the induction of iNOS in neuronal PC12 cells and that its cytotoxicity is mediated by the activation of JNK. [113]

The signal transduction pathway of growth factor withdrawal-induced apoptosis is discussed in the Chapter 1.

3.1.4. High density culture induced cytotoxicity

Plating a high cell density in the culture plate without increasing the medium volume when initiating the experiment creates an unfavorable condition for the culture. In such a culture model, cells are not supplied with sufficient nutrients, or growth factors, or even adhesion surface. A large number of cells die under such conditions, and the calcium, together with other toxic fragments released to the medium by ruptured cells may trigger further cell death. The current model at least partially mimics brain stroke or ischemia by maintaining a nutrient deficit condition in the culture. When ischemia occurs in the brain, a failure of cell membrane pumps is an early sign of neuronal injury while swelling of the Er and mitochondria will follow. [2] In the later stages, irreversible mitochondrial damage will occur, leading to a greater elevation in Ca^{++} influx, and subsequently resulting in cell death. In brief, brain ischemia triggers a complex cascade of molecular events. Identified mechanisms of postischemic neuronal injury include altered calcium homeostasis, free radical formation, mitochondrial dysfunction, protease activation, altered gene expression, and inflammation. [593]

The precise mechanism of neuronal death for this model is not fully understood. However, the similarity to brain ischemia gives this model a very attractive future for neurotoxicity research.

3.2. Experimental design

3.2.1. Determination of the culture conditions and time course

Cells, with or without treatment with Aβ, MPTP, and serum deprivation, were plated at multiple cell densities, ranging from ~400 cells/ml to 10^6 cells/ml, and then cultured for 48, 72, and 96hr. Cells from different treatment groups were harvested and assayed for viability after each designated time. A cell density of 3×10^4/ml and a time course of 96hr were chosen to investigate the cytotoxicity and neuroprotective effects in later experiments.

3.2.2. The effects of different insults in SK-N-SH cell culture

3.2.2.1. Aβ toxicity in SK-N-SH cell culture

Aβ 25~35 (20μM) was added to the culture media, and the cell density was 3×10^4 cells/ml. Cells were harvested after 96hr in culture, and assayed for cell viability using the WST-1 assay.

3.2.2.2. MPTP-induced cytotoxicity in SK-N-SH cells

MPTP (0.5, 0.75, or 1.0mM) was added to the cell culture media, and the experiment was carried out under the same conditions and with the same cell densities as for Aβ. Cell viability was assayed after 96hr.

3.2.2.3. Serum deprivation-induced neuronal death

Cells were cultured in serum-free-medium, or in medium with reduced FBS concentration (1%, 3%, or 5%). The culture period, cell density, and assay method were the same as described for the Aβ and MPTP models.

3.2.2.4. The cytotoxic effects of the high density culture model

Cells were plated at 2×10^5/ml in regular MEM when the experiment was initiated, and were assayed at 96hr of culture.

3.2.3. Distinguishing the type of cell death in all four models

To examine whether the neuronal death in the above cytotoxic models is necrotic or apoptotic, the ELISA quantification of mono- or oligonucleosomes and the detection of DNA laddering were used to identify apoptotic cell death. If apoptosis induction was low in any cytotoxic treatment groups, necrosis would be considered as induced by that insult. Characterization of cell death may help to further illustrate the cellular mechanisms for future neuroprotection studies. Moreover, cycloheximide, a protein synthesis inhibitor, was also used in this part of the experiment to further identify the apoptotic mechanisms of certain models. Apoptosis is a gene-regulated process, and involves protein synthesis; therefore, a protein synthesis inhibitor will show a protective effect in toxin-induced apoptotic neuronal death.

3.2.4. Identification of cytotoxicity vs. inhibition of proliferation

SK-N-SH cells proliferate under normal culture conditions. The apparent decrease of cell viability caused by each insult could be through two possible mechanisms; cytotoxicity, and inhibition of proliferation. To define the decreases in cell viability, cytosine-arabinoside (cArb), a selective inhibitor of DNA synthesis, was chosen to block cellular proliferation. The cArb efficacy has been related to the phosphorylation of cArb by deoxycytidine kinase and incorporation of cArbCTP into DNA during DNA synthesis, forcing the premature termination of DNA elongation, and leading to a termination of cell cycle. Thus, cell number would remain constant for the entire culture period. A further decrease in cell viability in any model would be the result of a cytotoxic effect. The effects of cArb was tested in SK-N-SH cells. Among the range of concentrations tested, cArb at concentrations greater than 1μM sufficiently blocked the proliferation (Fig. not shown). Aβ (20 μM) or MPTP (1mM) were added to the culture, and cytotoxicity was tested by assaying the cell viability after 96hr culture.

3.2.5. Role of kinases in cytotoxicity

In the current study, a group of protein kinase inhibitors were chosen to further distinguish the cytotoxicity induced by different models. These inhibitors included inhibitors of PKA, PKC, PKG, MAP kinase, and protein tyrosine kinase. Protein kinase inhibitors have provided much information about regulation and coordination of physiological functions. The inhibitors were chosen based on their well recognized functions in growth factor regulation and cell death pathways. The use of the kinase inhibitors can highlight different mechanisms in the above cell death models.

3.3. Results

3.3.1. The response of SK-N-SH cells to different insults

3.3.1.1. Aβ

To determine the optimal condition for the cytotoxic experiments in the current study, cell cultures were incubated for 48, 72, or 96hr in the respective treatment media with the initial cell densities ranging from about 400 cells/ml to 10^6cell/ml in 96 well plates. The toxic effects of Aβ on SK-N-SH cells were time course dependent and cell density dependent. SK-N-SH cells plated at densities greater than $3x10^5$ cells/ml did not show diminished cell viability at the 48hr, and the culture with cell densities of $1.25x10^5$ cells/ml or greater did not significantly affect the cell viability at 72hr and 96hr. The killing property of Aβ (20μM) was maximum at the 96hr culture period with the cell density ranging from 1×10^4 to 3×10^4 cells/ml (Fig. 3.1 to 3.3). A cell density of 3×10^4 cells/ml, and a time course of 96hr were chosen for further experiments.

3.3.1.2. MPTP

Based on the observation that Aβ induced severe cell death with 96hr culture, the MPTP (0.75mM) effect was investigated in SK-N-SH cells incubated for 96hr with cell densities of 3×10^4, 5×10^4/, 10^5 and 2×10^5 cells/ml. As in the Aβ experiment, the cytotoxicity of MPTP (0.75mM) was most servere at the 96hr culture period with the cell density in the range of 3×10^4 cells/ml (Fig3.4). Additionally, MPTP (0.5mM, 0.75mM, and 1.0mM) dose-dependently decreased cell viability (Fig. 3.5). The concentration of 0.75mM was chosen for further experiments.

3.3.1.3. Serum deprivation

Serum deprivation is a strong insult that induces severe cell death in SK-N-SH cells. SK-N-SH cell cultures were incubated for 48, 72, or 96hr in MEM containing 1% FBS with the initial cell densities ranging from about 400 cells/ml to 10^6cell/ml in 96 well plates. The 1%FBS culture also had a tendency to induce more cell death at lower cell densities, but the prolonged culture time did not seem to further influence cell viability (Fig. 3.6 ~ 3.8). Cells at a density higher than 10^5 cells/ml were not as sensitive serum withdrawn as they were with lower densities. Cell density at 3×10^4 cells/ml, and a culture period of 96hr were chosen to perform further experiments.

3.3.1.4. High density

In addition, it has been observed that under regular culture conditions, a plated cell density greater than 10^5cells/ml will induce a significant cell death in a time-dependent manner, and the severity of cell death parallels the initial cell density (Fig. 3.1 to 3.3, 3.6 to 3.8). Based on this particular phenomenon, a high density model was established with a time course of 96hr cell culture and a cell density of $2x10^5$ cells/ml.

After establishing the culture conditions for each individual cytotoxic model, the cytotoxicity was investigated and analyzed. Aβ (20μM) significantly reduced cell number by 33.28 ± 2.89 % (Fig. 3.9) compared with the control group after 96hr of culture with an initial cell density of $3x10^4$ cells/ml, and MPTP (0.75mM) significantly reduced cell number by 37.56 ± 3.35 % (Fig. 3.10). Serum-free medium decreases the cell viability by 92.15 ± 0.96 % (Fig 3.11). In the presence of FBS, cell viability was restored proportionally to the concentration of FBS; compared with serum-free medium, 1% and 3% FBS decreased cell viability by 73.07 ± 2.21% and 49.67 ± 3.65 %, respectively, and 5% FBS maintained cell survival after 96hr of culture (Fig. 3.11). In the high density model, due to multiple factors that were not suitable for the culture, only 18.55 ± 1.03% of the suspended cells survived after 96hr of culture (Fig. 3.12).

3.3.2. The detection of apoptosis in the SK-N-SH cells

To characterize the type of cell death, an ELISA quantification of histone-bound mono- or oligonucleosomes and a DNA ladder gel were performed.

3.3.2.1. Cell death detection ELISA – oligonucleosome accumulation

In a regular cell population under normal culture conditions, 3% to 8% of cells may undergo apoptosis; [483] therefore, a low reading in the control group is expected.

In dying cells, different nuclear events are associated with apoptotic and necrotic cell death. Aβ (20μM) was found not to induce apoptosis in SK-N-SH cells after 96hr culture, since it failed to induce any stronger apoptotic signal compared with control cells (Fig. 3.13). Following 96hr exposure to MPTP (0.5 to 1.0mM), a dose-dependent increase in mono- or oligo-nucleosome accumulation (Fig. 3.13) was evident in the cytoplasmic fraction of cell lysates, indicating the cell death induced by MPTP in SK-N-SH was through apoptotic pathways. The serum-free model showed a greater than100 fold increase in nucleosome accumulation, and 1% and 3% FBS also induced significant apoptotic neuronal death after 96hr culture (Fig. 3.14). The results suggest that growth factor withdrawal, either completely or partially, is a strong insult to induce apoptosis which is probably through the Ras signaling, immediate-early gene (IEG) pathway revealed by Mesner. [400] The high density model was also found to induce nucleosome accumulation after an incubation of 96hr in culture, suggesting that nutrient deficiency in this model may be an apoptotic insult, as well (Fig. 3.14).

3.3.2.2. DNA ladder gel

DNA laddering is one of the most well established methods for identifying apoptosis. When apoptotic cell death occurs, the endonuclease selectively cleaves at the linker DNA, generating DNA fragments which are multiples of approximately 180bp in length, and upon agarose gel electrophoresis, a ladder pattern will be evident. Individual bands of the ladder represent oligonuclesomes of different lengths produced by the internucleosomal cleavage of nuclear DNA. In contrast, nuclear DNA in necrotic cells remains mainly intact or becomes randomly degraded in severely damaged cells, resulting in DNA fragments of heterogeneous length, and will appear as a smear on the agarose gel. Among all the cytotoxic models, DNA extracted from the MPTP (0.75mM), high density and serum-free-treated cell culture were clearly internucleosomally fragmented as judged by the DNA laddering, suggesting that the cell viability decrease in these models was due to apoptotic neuronal death. The DNA laddering in the Aβ (20μM) model, as in the nuclesome ELISA experiment, did not differ from that of the control group (Fig. 3.15). These findings correlated with observations on oligonuclesome quantification, and confirmed that at 96hr, Aβ, in this cell line, simply induces necrosis; while at 96hr, MPTP, high density and serum deprivation models of cell death are through certain apoptotic pathways.

3.3.2.3. The effect of cycloheximide in Aβ and MPTP models

Cycloheximide is a protein synthesis inhibitor in eukaryotes. A dose-response experiment with cycloheximide (0.1 to 100μg/ml) showed that at a concentration of 3μg/ml, cycloheximide sufficiently inhibits proliferation by inhibition of protein synthesis in SK-N-SH cells (data not shown). Protein synthesis is essential for mitosis. Inhibition of protein synthesis will terminate the cell cycle by inhibiting the translation process, and subsequently inhibits cell proliferation. The experiments with cycloheximide were only carried out in the Aβ and MPTP models, as these two models are closely related to neurodegenerative changes, and represent different types of cell death as determined by oligonucleosome accumulation and DNA laddering. When Aβ (20μM) was added to cycloheximide-treated cells, it further decreased cell number by 29.36 ± 2.29 %. MPTP (0.75mM) did not change the cell viability in cycloheximide-treated culture (Fig. 3.16). Thus, cycleheximide had a neuroprotective effect in the MPTP cytotoxic model. Since apoptosis is a well regulated biological process which involves gene regulation and protein synthesis, inhibition of protein synthesis will interfere with apoptotic pathways. The above evidence further suggested that the MPTP model acts through apoptotic cell death.

3.3.3. Identification of cytotoxicity *vs.* inhibition of proliferation

In serum-free and high density models, the cell numbers assayed after 96hr culture were lower than the initial cell density, indicating a cell death process occurred in these two models. To further characterize the cell death in Aβ and MPTP models, cArb was added to cell culture to inhibit proliferation. cArb (10^{-6}M) maintained the cell number at the plated density after 96hr culture (Fig.3.17), and the addition of Aβ (20μM) and MPTP (1.0mM) decreased cell viability by 34.46 ± 5.14 %, and 26.27 ± 4.17 %, respectively, compared with cArb-treated SK-N-SH cells (Fig. 3.17). The results revealed that cell viability decrease in these two models occurred at least partially through cytotoxic effects. However, it could not be concluded that the cytotoxicity was responsible for the total viability decrease in each model. Probably, in some of the models, the inhibition of the cell proliferation contributed partially to the cell number losses.

3.3.4. Further characterization of the cytotoxic models

Cell death, either apoptosis or necrosis, involves dysregulation in various signal transduction pathways. Cellular signalling cascades rely on the phosphorylation status of proteins in their pathways. Gene expression, cell cycle progression, and differentiation of cells are controlled by the complex interplay of protein kinases in specific signalling pathways. In the current study, several inhibitors of protein kinases were chosen in order to identify which kinase may play a role in each of these cytotoxic models. The non-specific protein kinase inhibitor, H7, the PKA inhibitor 14-22 Amide, the PKG inhibitor, KT5823, and the protein tyrosine kinase inhibitor, AG18 did not show any effect in these cytotoxic models (data not shown). On the other hand, the MAP kinase inhibitor, SB203580 (1μM), successfully inhibited the viability loss caused by Aβ, but not other insults (Fig. 3.18). The selective PKC inhibitor, chelerythrine (5μM), although somewhat cytotoxic in the control group, and Aβ- and MPTP-treated cultures, strongly reversed the high density-induced cell number decrease (Fig. 3.19). These results suggest that each of the 4 cytotoxic models has its unique cytotoxic pathway; moreover, although MPTP, high density, and serum deprivation all induce apoptosis, the apoptotic pathway for each model involved is different.

3.4. Discussion

SK-N-SH is a neuronal cell line which was developed by J.L. Biedler *et al.*, [55] and is widely and extensively used as a target cell line in cell-mediated cytotoxicity assays. The cell line was established in cell culture from human metastatic neuroblastoma tissue and maintained *in vitro*. SK-N-SH consists of two morphologically distinctive cell types, a small spiny cell and large epithelioid cells. In monolayer culture, the cell line forms a disoriented growth pattern and reaches high saturation densities. The population-doubling time is about 44hr. SK-N-SH cells have been characterized by high dopamine-β-hydroxylase activity. A very low activity level of glutamic acid decarboxylase, the enzyme responsible for the conversion of glutamate to GABA, has also been found in this cell line. Choline acetyltransferase activity in this cell line is negligible. [56] These findings indicate that SK-N-SH cells exhibit a neuronal phenotype, and have multiple neurochemical markers. In addition, based on the observed responsiveness of SK-N-SH cells to the Aβ, MPTP, serum deprivation and high density insults, it can be concluded that this cell line is very useful in the assessment of neurotoxicity and neuroprotection.

The current study has shown Aβ-induced neuronal cell death in SK-N-SH cells, which confirms the previous report of Green *et al.* [225] They observed that four days of exposure of SK-N-SH cells to Aβ 25~35 resulted in a dose-dependent loss of cell viability with a LD_{50} of 28.9μM as illustrated by the trypan blue exclusion method. [225] Aβ reduced cell number by 36, 65, 70, and 83% at the respective dose levels of 10, 20, 40, and 80μM. In another study, they reported an acute toxic effect with Aβ. The addition of Aβ (20μM) reduced cell number by 5% in 3hr to a maximum of 35% at 15hr. [232] The time course of the Aβ toxicity in SK-N-SH cells is similar to that reported for primary cortical neurons. [619] The cell density-dependence of Aβ in the current studies is proposed to be involved in "cell to cell communications": the lower the cell density, the poorer cell to cell communication. Toxic insult may amplify its cytotoxicity in such unfavorable culture conditions. Altered signal transduction and aberrant kinase activities have been proposed to contribute to Aβ toxicity. Among the MAP kinases, p44 MAPK (44 kDa; extracellular signal-regulated kinase 1; ERK1) and p42 MAPK (42 kDa; ERK2) play a pivotal role in the mediation of cellular responses to a variety of signaling molecules. [494] A number of growth factor receptor tyrosine kinases are known to be linked to the G-protein Ras and to stimulate MAPK kinases such as Raf. These kinases phosphorylate and activate MAPK/ERK kinases 1 and 2 (MEK1/2), which in turn phosphorylate and activate ERK1/2. The phosphorylated ERK1/2 translocates into the nucleus and plays a role in the regulation of gene transcription. In this way, Ras, Raf, MEK1/2 and ERK1/2 constitute a unique signal transduction cascade. Rapoport [476] has reported that Aβ induced a sustained increase in ERK phosphorylation in mature hippocampal neuron cultures. The effect of Aβ on ERK in neurons is still controversial. We have found that SB203580 (1μM), a p38 MAP kinase inhibitor, restored the cell loss induced by Aβ, suggesting that in SK-N-SH cells, the cytotoxicity of Aβ is through the dysregulation of the p38 MAPK cascade. These findings correlated with previous observations that Aβ reduced basal synaptic transmission in rat hippocampal slices, and inhibited the long term potentiation (LTP) upon stimulation. In the presence of SB203580, Aβ failed to give rise to a reduction in LTP, suggesting that the toxicity of Aβ in synaptic transmission may inhibit LTP through a pathway involving the MAP kinases. [494] These observations suggest that Aβ dysregulates MAPK cascades through both ERK and p38 MAK pathways. The inhibitors for other kinases tested in the current study, including H7, the PKA inhibitor 14-22 Amide, chelerythrine, KT5823, and AG18 did not show any effect in the Aβ model, suggesting that probably the Ras/Raf, MAPK cascade, but not other upstream pathways, is unique in Aβ cytotoxicity in SK-N-SH cells. However, Ekinci *et al.* [164] have reported that Aβ did not affect ERK phosphorylation in SH-SY-5Y human neuroblastoma cells and primary cultured mice cortical neurons. This difference may be due to cell line or tissue specificities.

By using the oligonucleosome ELISA-quantification and DNA laddering methods, we concluded that the Aβ-induced cytotoxicity is through a necrotic pathway. However, amyloid protein-induced apoptosis has been observed with primary cortical and hippocampal culture, and with a few other cell lines. This process is considered to be a unique hallmark and mechanism of AD. [79, 119, 150, 256, 300, 613, 624] On the other hand, other reports indicated that Aβ caused a necrotic type of neuronal death in some other cell culture models. [38, 219, 546] Geschwind and Huber [240] summarized several studies and concluded that apoptotic cell death induced by Aβ peptide was cell type dependent. There is abundant information concerning neurocytotoxicity in the currently available literature. Research on Aβ in the past decade has revealed that Aβ induces cell death through a complex cascade at the cellular and molecular level. These aspects were reviewed in the introduction to this chapter. Much of the conflicting evidence may be explained by "cell type dependence".

There have been multiple lines of evidence supporting a decline in glucose metabolism in early AD. [245, 531] Decreased concentrations of glucose transporters have been noted in the brains of AD patients. [131, 384] Selective decreases in the expression of mitochondial enzymes necessary for oxidative metabolism have been reported. [315] It has also been noted that Aβ-treated SK-N-SH cells showed increased consumption of glucose in close association with cell death. A 78% increase in lipid peroxidation was recorded, as well. [232] The phenomenon was proposed to allow for repair and maintenance of membrane integrity and it also suggests ROS production in response to Aβ, and that the toxicity of Aβ is at least partially due to the oxidative stress.

The discovery of the dopaminergic toxin MPTP provided a specific example of how an exogenous toxic substance can initiate a PD-like syndrome and provide a model for studying cell death in PD. In the current study, MPTP (0.75mM) induced a 37.56% loss of cell viability. Since SK-N-SH cells express relatively high dopamine hydroxylase activity, it is reasonable that the dopaminergic toxin has a clear toxic effect in this cell line. Moreover, the results from the oligonuclesome ELISA and the DNA laddering analysis indicated that the cell death induced by MPTP was through an apoptotic pathway. It has also been observed that in the presence of cycloheximide, a protein synthesis inhibitor, MPTP did not show a cytotoxic effect in SK-N-SH cells. This further supports the idea that MPTP cytotoxicity is an apoptotic process, since gene regulation and protein synthesis are essential steps in apoptosis. There has been abundant information indicating that MPTP toxicity in nigral dopaminergic neurons contributes to PD in humans, [338, 479, 570] monkeys, [105, 308, 341] and some rodents. [162, 284] Previous work has indicated that MPP^+, the active metabolite of MPTP, induced apoptosis in cultures of human SH-SY5Y neuroblastoma cells. [214] Nuclear fragmentation, DNA laddering, and a 20% decrease in viability were seen after a 4-day incubation with 5 μM MPP^+. The authors observed that MPP^+-induced apoptosis was completely prevented by the nonselective caspase inhibitor zVAD.fmk but not by the caspase-8 inhibitor IETD.fmk. De Girolamo and co-workers [133] showed that subcytotoxic concentrations of MPTP inhibited axon outgrowth and were associated with increased neurofilament heavy chain (NF-H) phosphorylation in differentiating mouse N2a neuroblastoma cells while higher doses (> 100μM) caused cell death. The neurotoxic effects of MPTP occurred in the absence of significant alterations in energy status or mitochondrial membrane potential. The p38 MAP kinase inhibitor, SB202190, protected cells against MPTP-induced neurotoxicity at a certain range of MPTP concentrations (1.0 ~ 10μM), but it could not maintain cell viability at high MPTP (>100μM) concentrations. [133] The observation with a MAP kinase inhibitor conflicts with what has been detected in our SK-N-SH cells, where the SB230580 did not change the cell viability decreased by MPTP. The difference between these experiments is very likely due to the difference of the culture system tested, for each cell line may respond to different insults through its unique signal transduction pathways.

MPTP administration to primates and mice can replicate the selective degeneration of neurones, thereby mimicking the clinical and pathological features of PD. The cytotoxicity induced by MPTP is identified as apoptosis in different experiments with different systems.[143, 163, 214, 472] MPTP exerts its neurotoxic effect through biological activation by the enzyme MAO-B, producing an unstable intermediate 1-methyl-4-phenyl-2,3-dihydropyrdinium ion ($MPDP^+$), prior to forming the toxic metabolite MPP^+. It is known that MPTP can inhibit mitochondrial respiration via complex I, leading to mitochondrial energy deprivation and eventual cell death. In addition, MPTP is thought to mediate the generation of toxic ROS, causing lethal damage to critical biomolecules. This is supported by findings that the protective capacity of various antioxidants alleviates MPTP-induced cell death. These discoveries have greatly influenced PD research in identifying possible targets for pharmacological intervention. A detailed mechanism of MPTP-induced apoptosis is discussed in the Introduction (chapter 1). However, the exact mechanisms of MPTP-induced dopaminergic damage are still not entirely understood.

Serum deprivation is a well established model in apoptosis research. We found that complete or partial removal of FBS can cause a severe loss of live cells in SK-N-SH cell culture after 96hr. This confirms the observation of Bishop *et al.*[63] that serum-free media decreased the thymidine uptake in SK-N-SH cells. The loss of viability in our study is due to apoptotic cell death as detected with oligonuclesome ELISA quantification and DNA laddering methods. Many reports have indicated that apoptosis is induced by serum withdrawal in multiple neuronal cultures. For instance, cerebellar granule neurons of mouse and hippocampal neurons of rat embryo exhibited increased apoptosis when cultured in serum-free medium. [17, 630] A proportion of differentiated SH-SY5Y cells died in response to withdrawal of serum. This death manifested the hallmark features of apoptosis including changes in nuclear morphology, processing and activation of caspase 3 and cleavage of the caspase 3 substrates. [97] SH-5Y5Y cell survival is dependent on the continuous presence of brain-derived neurotrophic factor, and removal of this neurotrophin causes apoptotic cell death. Rat cortical neurons died in an apoptosis-like and a caspase-independent manner under serum-free conditions. [249, 261] Fibroblast growth factor (FGF) 2 is a neuroprotective factor for various cell types. FGF2 protective activity was studied in chick retinal neurons subjected to apoptosis by serum deprivation. Exogenous FGF2 supported neuronal survival after serum deprivation and increased neuronal *bcl-xl* and *bcl-2* expression, through binding to its receptor R1 (FGF-R1), and subsequent ERK activation. [142] Recent studies suggest that hepatocyte growth factor (HGF) functions as a neurotrophic factor in the central nervous system. Cultured cerebellar granule neurons underwent apoptosis when the culture medium was changed from that containing serum with 25 mM K^+ to serum-free medium containing 5 mM K^+, and HGF prevented the apoptotic cell death. [630]

Serum deprivation-induced apoptosis is usually through growth factor withdrawal. In general, growth factor withdrawal induces JNK activation and the phosphorylation of *c-Jun*, which in turn induces the expression of DP5/Hrk, a "BH3-domain only" member of the *Bcl-2* family. DP5 may activate *Bax*, causing mitochondrial damage, which results in the release of cytochrome *c*. Formation of the cytochrome *c*/Apaf-1/caspase-9 complex induces the activation of caspase-9. Activated caspase-9 in turn activates caspase-3, leading to apoptosis. A lack of trophic factor signalling also induces a non-nuclear competence-to-die pathway that facilitates the formation of the cytochrome *c*/Apaf-1/caspase-9 complex, resulting in caspase activation. [272]

Neuronal injuries and neuroprotection are key topics in neuroscience research. A reliable *in vitro* cytotoxicity model in such a research area is essential. The current cytotoxic system allows the study of neurotoxicity and "neuroprotection" under conditions where the different pathways and mechanisms of the neurons can be considered within one system, removing variations which may be due to different systems studied.

Few diseases can be as devastating as those related to the damage of the nervous system following excitotoxic insults such as the global ischemia of cardiac arrest, the focal ischemia of a stroke or a major-seizure disorder. For these reasons, a tremendous amount of research has focused on the mechanisms underlying excitotoxic neuron death, and out of this has come a cascade that is usually related to similar groups of insults. Such insults involve synaptic accumulation of excessive glutamate, which, at sufficiently high levels, becomes neurotoxic. This is primarily mediated by the mobilization of $[Ca^{++}]_i$ in the postsynaptic neuron. This, in turn, leads to the generation of free radicals, energetic collapse, damage to the cytoskeleton, protein misfolding and, in a subset of neurons, the triggering of apoptosis. [343] However, few of the available models are suitable for stroke and brain ischemia studies. The setup of the high density model could be a good system for *in vitro* studies of stroke, since it mimics certain aspects of the ischemic condition, including nutrient deprivation, failure to remove metabolic waste, energy depletion, and oxygen insufficiency. Although the exact cellular mechanism of this model is not fully understood, the observation of severe cell viability loss, a strong apoptotic signal, and the involvement of PKC regulation imply that the cell death in this model could be tightly involved in oxidative stress and mitochondrial dysfunction. In addition, it is demonstrated that the neurite outgrowth of neuroblastoma cells is influenced by alterations in signal transduction protein kinases, including induction by PKC inhibition. [132, 133] Thus, the high density model has further potential in neurotoxic research as related to both cytotoxicity and differentiation.

Development of neuroprotective therapies for multiple injuries or disease is dependent on defining the precise mechanisms whereby molecules or toxins are able to induce relatively selective injury of specific neurons. The selectivity of this injury could be conferred either by the properties of the effectors or the targets. The former reflects the neurotoxins in our case. Target related selectivity could reflect the expression of death-inducing surface receptors (such as Fas or TNFR-1) required for interaction with effector mediators and subsequent intracellular signaling pathways, including the caspase cascade and more. Development of therapeutic delivery systems, which would reach the site of disease activity within the CNS, will permit the administration of inhibitors either of the cell death pathway or of effector target interaction and may open new avenues to approach neuroprotection. [500]

The available *in vitro* cytotoxic studies have, to date, provided tremendous information, but there remain many conflicting observations. The difference in defining whether Aβ is an apoptotic insult in different studies is an example of such conflicts. For another example, K^+ channel function may be involved in apoptosis. Chi *et al.* [110] indicated that the activities of K^+ channels on cerebellar neurons decrease during apoptosis. When K^+ channel blockers were investigated, it was found that 4-Aminopyridine (4-AP) blocks an outward rectifier K^+ channel and causes apoptosis in malignant astrocytoma cell lines. Clofilium, a K^+ channel blocker, induces apoptosis of human promyelocytic leukemia (HL-60) cells via *Bcl-2*-insensitive activation of caspase-3. [111] K(ATP) channel openers are able to provide hippocampal neuroprotection, and K(ATP) channels are considered to be predictive of neuronal survival.[70] Interestingly, while K^+ channel blockage elicits an apoptotic response in some cells lines, tetraethylammonium (TEA), the quaternary ammonium ion and nonselective K^+ channel blocker, is protective against neuronal apoptosis in cortical culture. [586] Therefore, these observations yield confusing conclusions regarding whether up-regulation or down-regulation of K^+ channel function can be either neurocytotoxic or neuroprotective. The conflicting results regarding apoptosis in the above investigations are probably due to differences in the setup of their cytotoxic models. Whether an insult will induce apoptosis is cell line or cell culture condition-dependent. This could be very reasonable, because in the nervous system, each category of neurons has its unique characteristics, neurotransmitters, receptors, etc. Therefore, the signal transduction pathways regulating cell death or neuroprotection will vary from one cell line to another, from one category of neurons to another. Interpreting results of a particular insult from different studies which involve different cell lines or tissue preparations may lead to a misunderstanding of the true mechanisms, since the presumed multiple regulation cascades are probably simply due to the differences in cell line or tissue specific responding to the same insult. However, with the cytotoxic system in the current studies, the results that were observed in all models were reliable and comparable since tissue specificity was not interfering with the results.

There are several advantages of the cytotoxic models studied here. First, all the models are set up with one cell line. Therefore, cell specificity variance will not be a problem. Second, these models involve both apoptotic and necrotic cell death, which are the target points in neurotoxic research. Moreover, with the apoptotic models, there are different pathways that regulate each individual model. The multiple cascades of signal transduction pathways that lead from receptors on the cell membrane to the nucleus, thus translating extracellular signals into changes in gene expression, may represent important targets for neurotoxic and neuroprotective compounds. Among the biochemical steps and pathways that have been investigated are the metabolism of membrane phospholipids, and the modification of a multitude of protein kinases. Additionally, the cell viability decreases in each model have been considered as cytotoxic effects, since the assayed cell numbers were lower than the initial plated cell number in high density and serum-free groups. In the Aβ and MPTP groups, a decrease in viability has been recorded in the presence of cArb. However, it could not be concluded that the cytotoxic effects account for the total decrease in cell number. An effect of inhibiting proliferation can be another possibility, probably due to the changes in cellular metabolism and decreased energy level induced by the cytotoxicity, and diminished cell division. Thus, the current cytotoxic systems are of greater significance for investigation of the simultaneous both neuroprotection and neurotrophic effects.

A review of the available literature for neurotoxic and neuroprotective studies reveals that the causes of neuronal cell death could be summarized as oxidative stress and free radical accumulation, mitochondrial dysfunction and imbalanced calcium homeostasis. Among these, disruption of calcium homeostasis is a key factor for cellular functions and cell death. Detailed discussions about cellular calcium and neuronal functions, calcium homeostasis and cell death are provided in the Introduction (Chapter 1). A set-point hypothesis [182] clearly illustrates the relationship between cellular Ca^{++} and neuronal survival. The hypothesis poses a few set points of $[Ca^{++}]_i$ that influence neuronal survival. At one extreme is $[Ca^{++}]_i$ that is too low to support essential Ca^{++} dependent processes, and at the opposite extreme is markedly elevated $[Ca^{++}]_i$ that activates various destructive processes within cells. Calcium levels at these extremes are incompatible with neuronal survival. Evidence supports the notion that large elevations of $[Ca^{++}]_i$ mediate the death of neurons in response to excitotoxic or other insults. [112, 386] Very low levels of $[Ca^{++}]_i$ are detrimental to neuronal growth and survival. [182] Studies on calcium homeostasis are to be included in the future aspects of the current project. Within the current cytotoxic system, all listed causations for neuronal death could be relevant. Research on how protective agents manipulate these parameters should reveal the cellular mechanisms of certain neuroprotective pathways.

In conclusion, well established neurocytotoxic models in the current study, which include Aβ-, MPTP-, growth factor withdrawal-, and nutrient deprivation-induced neuronal death, can be useful systems for neurotoxic and neuroprotective research. These systems cover a broad range of cell death pathways induced by multiple insults, including both apoptosis and necrosis. In addition, the clinical relevance of these models cannot be ignored. An ideal protective agent which shows promising protective effects on any model can be investigated for its therapeutic effects on related neurodegenerative diseases or brain injury.

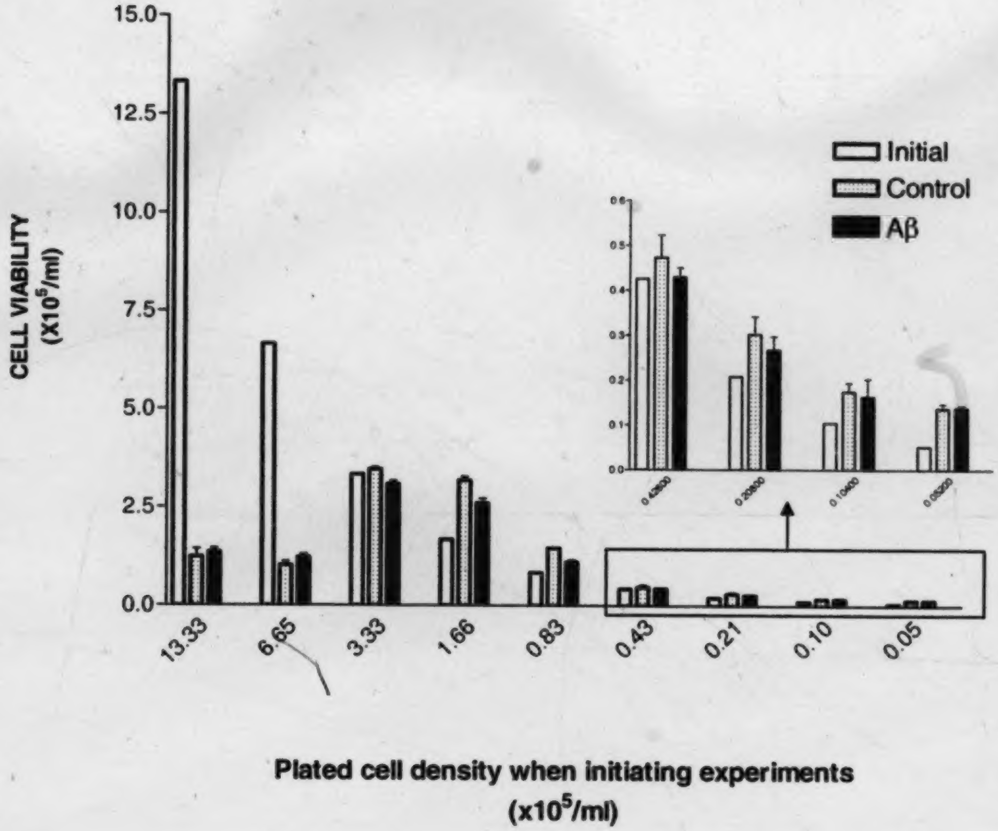


Fig. 3.1. The effects of Aβ (20μM) on a 48hr SK-N-SH cell culture.

Cells were suspended in the densities indicated on the X axis, and the cell viability was assayed after 48hr of culture.

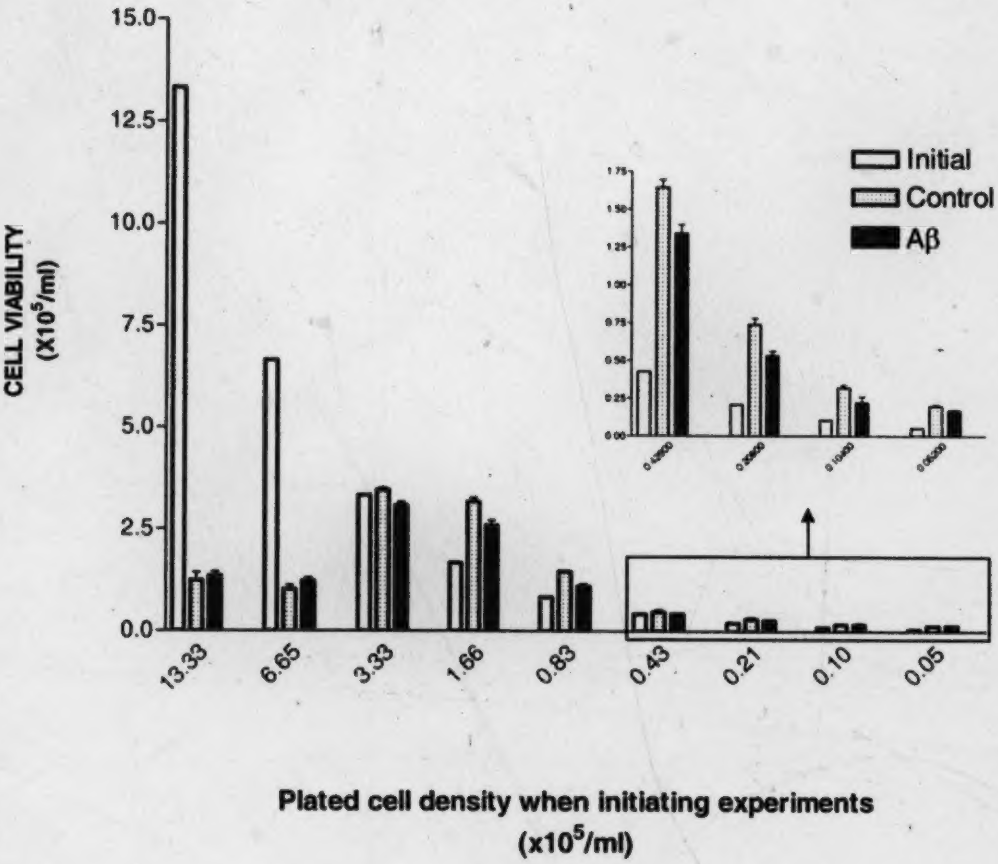


Fig. 3.2. The effects of Aβ (20μM) on a 72hr SK-N-SH cell culture.

Cells were suspended in the densities indicated on the X axis, and the cell viability was assayed after 72hr of culture.

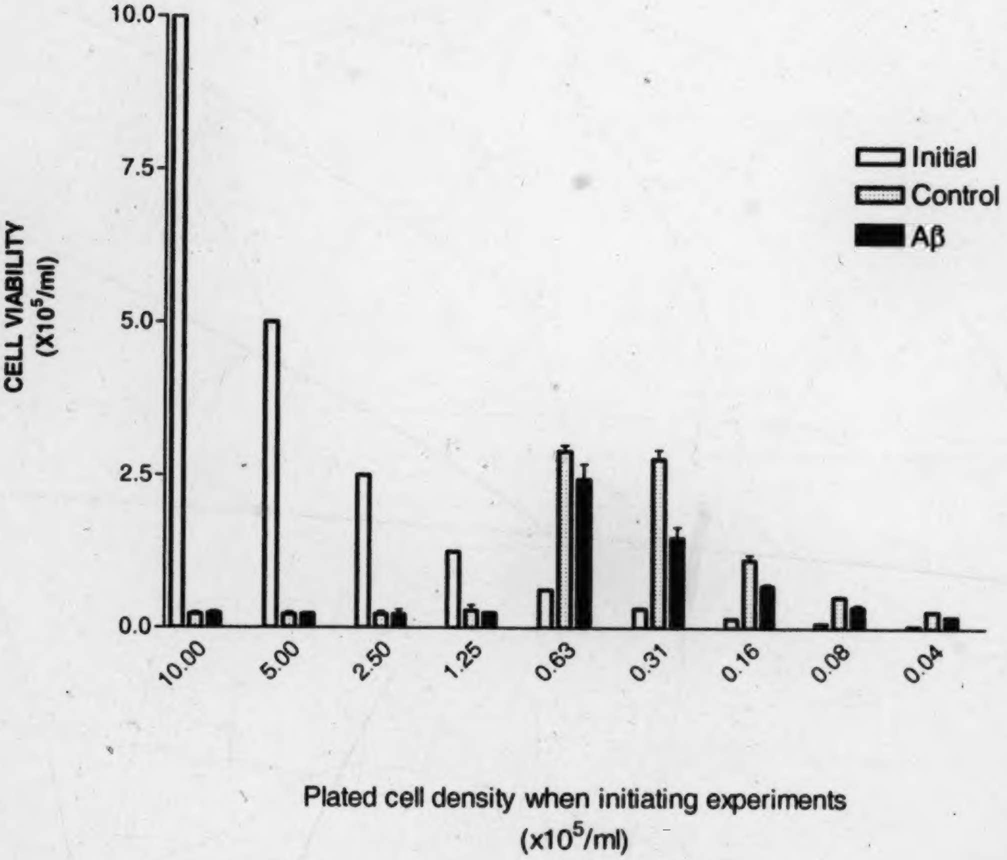


Fig. 3.3. The effects of Aβ (20μM) on a 96hr SK-N-SH cell culture.

Cells were suspended in the densities indicated on the X axis, and the cell viability was assayed after 96hr of culture.

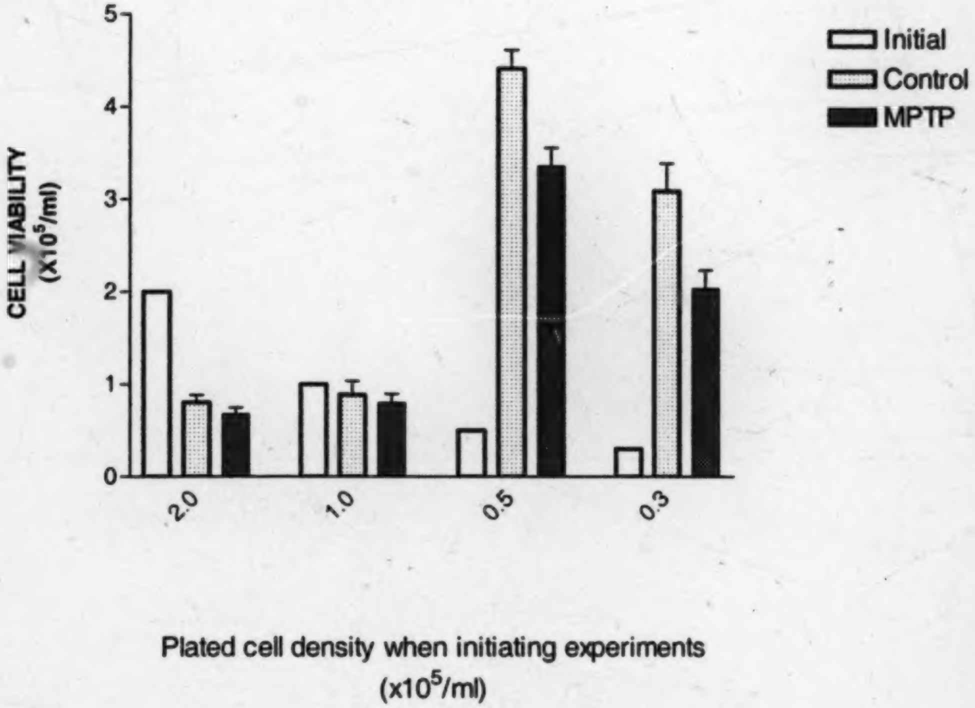


Fig. 3.4. The effects of MPTP (0.75mM) on a 96hr SK-N-SH cell culture.

Cells were suspended in the densities indicated on the X axis, and the cell viability was assayed after 96hr of culture.

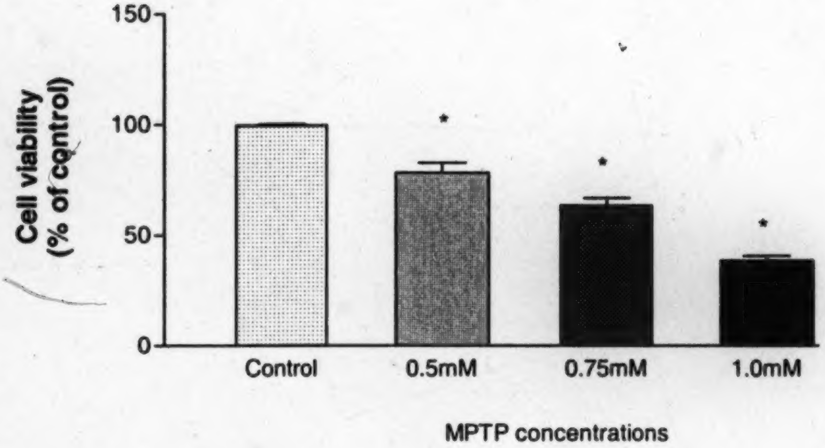


Fig. 3.5. The dose-dependent effects of MPTP (0.5, 0.75, and 1mM) on a 96hr SK-N-SH cell culture.

Cells were suspended in the densities indicated on the X axis, and the cell viability was assayed after 96hr of culture.

*, $p<0.05$; when compared with the control group. (n=4)

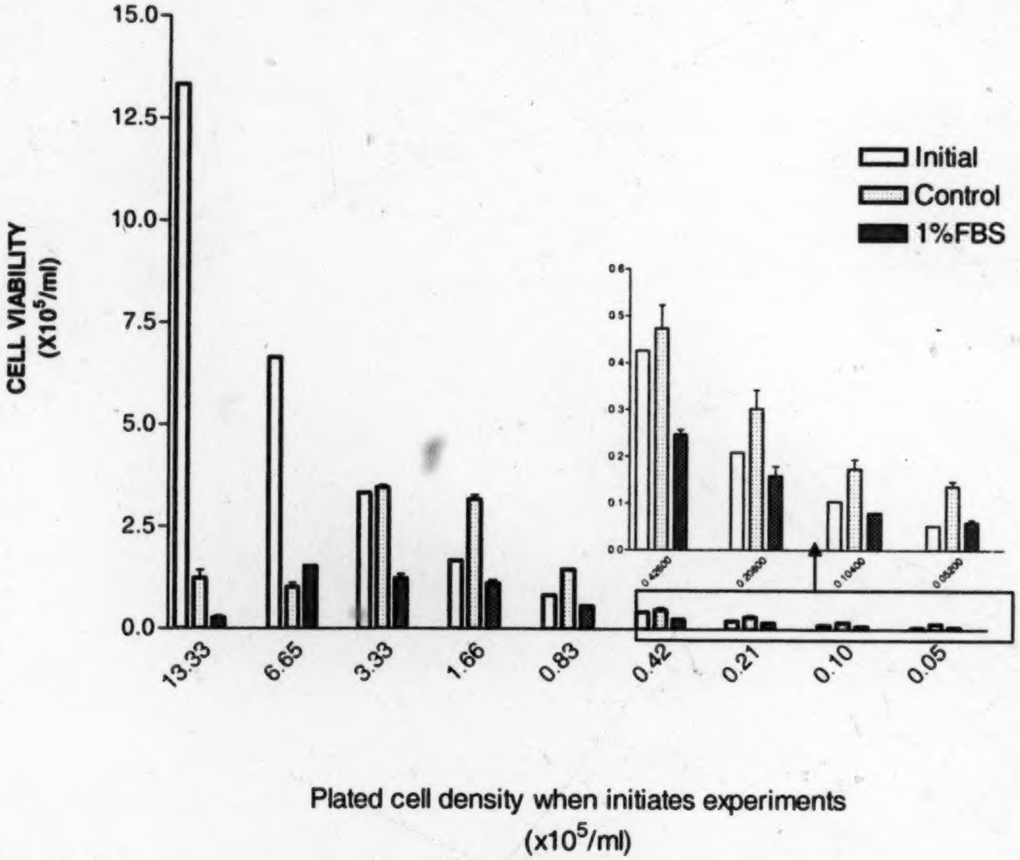


Fig. 3.6. The effects of 1% FBS on a 48hr SK-N-SH cell culture.

Cells were suspended in the densities indicated on the X axis, and the cell viability was assayed after 48hr of culture.

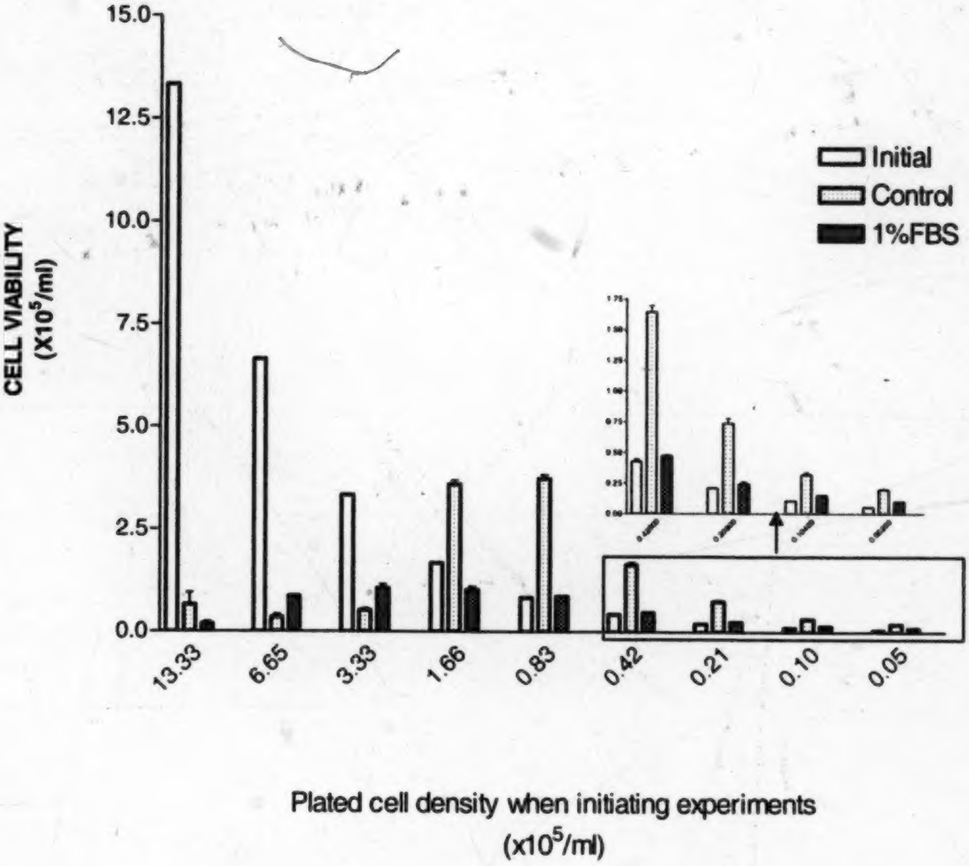


Fig. 3.7. The effects of 1% FBS on a 72hr SK-N-SH cell culture.

Cells were suspended in the densities indicated on the X axis, and the cell viability was assayed after 72hr of culture.

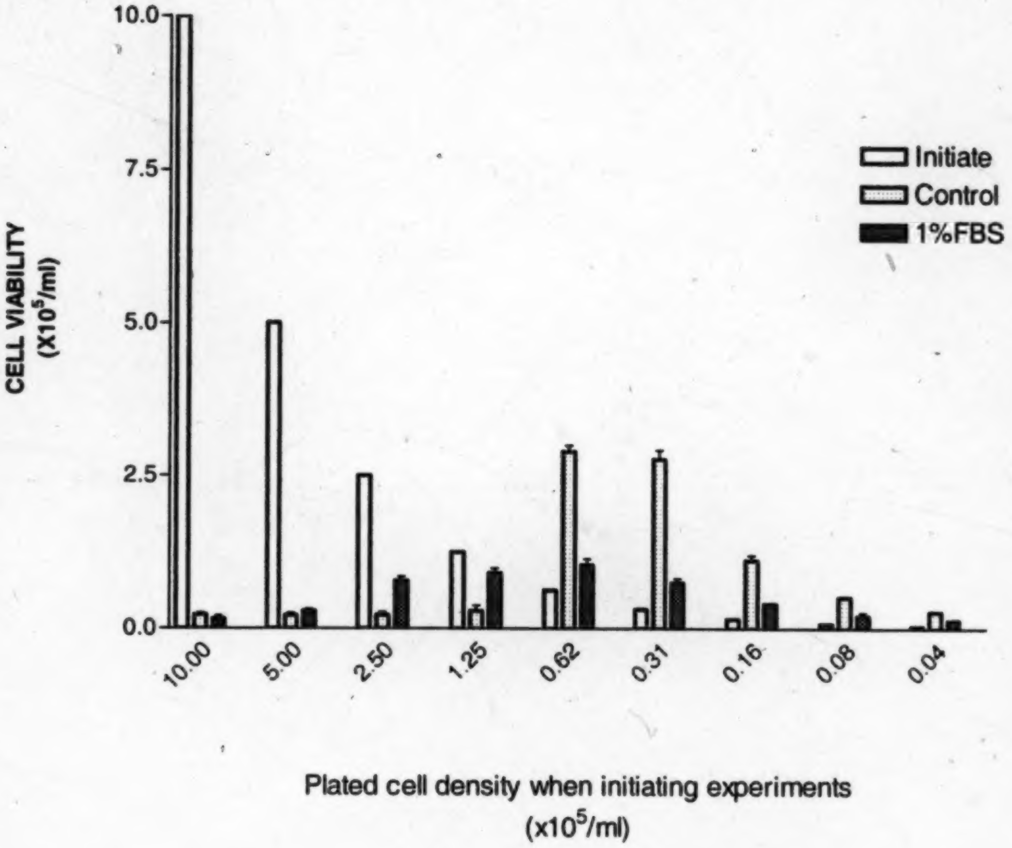


Fig. 3.8. The effects of 1% FBS on a 96hr SK-N-SH cell culture.

Cells were suspended in the densities indicated on the X axis, and the cell viability was assayed after 96hr of culture.

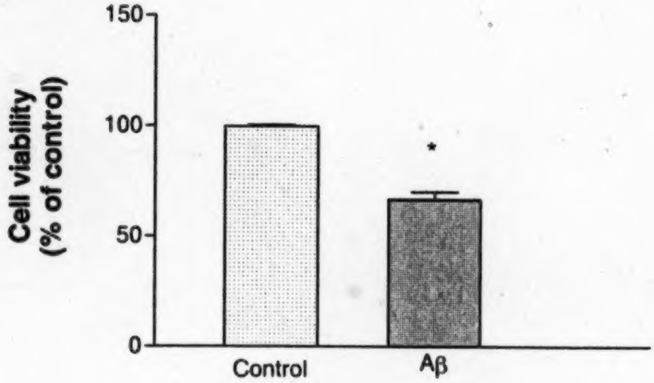


Fig. 3.9. Analyzed results of Aβ (20μM) cytotoxic effects on SK-N-SH cells in 96hr culture.

Cells were suspended at 3×10^4 cells/ml, with a total volume of 100μl /well in 96 well plate. Aβ was added to the cell culture when initiating experiments. Cell viability was assayed after 96hr.

*, $p < 0.05$, when compared with the control group. (n=8)

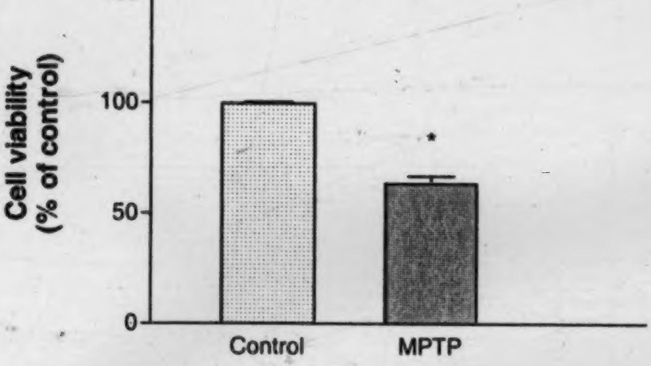


Fig. 3.10. Analyzed results of MPTP cytotoxic (0.75mM) effects on SK-N-SH cells in 96hr culture.

Cells were suspended at 3×10^4 cells/ml, with a total volume of 100μl /well in 96 well plate. MPTP was added to the cell culture when initiating experiments. Cell viability was assayed after 96hr.

*, $p<0.05$, when compared with the control group. (n=6)

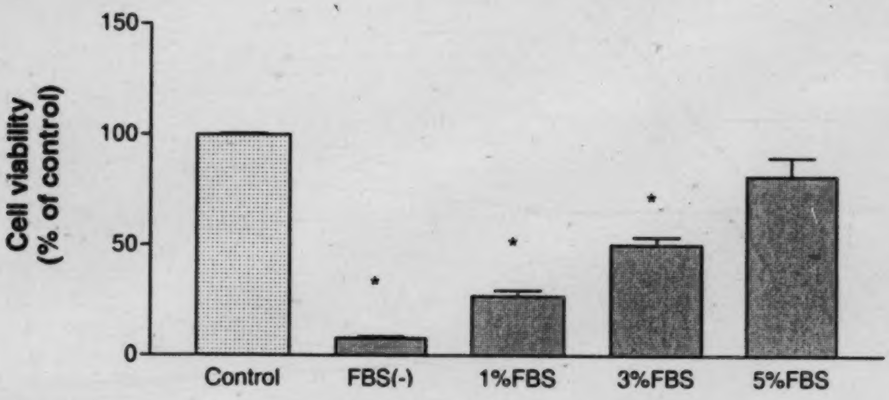


Fig. 3.11. Analyzed results of serum deprivation-induced cytotoxicity in SK-N-SH cells in 96hr culture.

Cells were suspended at 3×10^4 cells/ml, with a total volume of 100µl /well in 96 well plate. The media with reduced or deprived FBS were used in the treatment groups. Cell viability was assayed after 96hr.

*, $p<0.05$, when compared with the control group. (n=5)

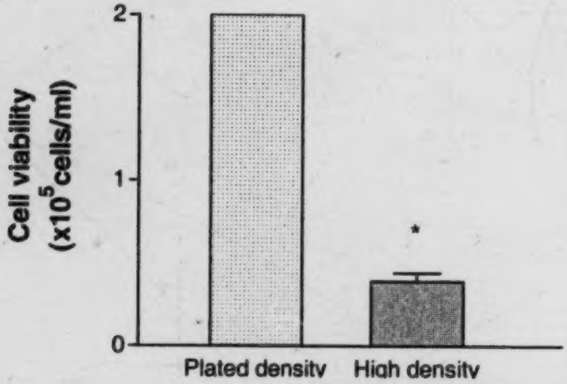


Fig. 3.12. Analyzed results of the effects of high density culture on SK-N-SH cells in 96hr culture.

Cells were suspended at 2×10^5 cells/ml, with a total volume of 100μl /well in 96 well plate. Cell viability was assayed after 96hr.

*, $p<0.05$, when compared with the control group. ($n=6$)

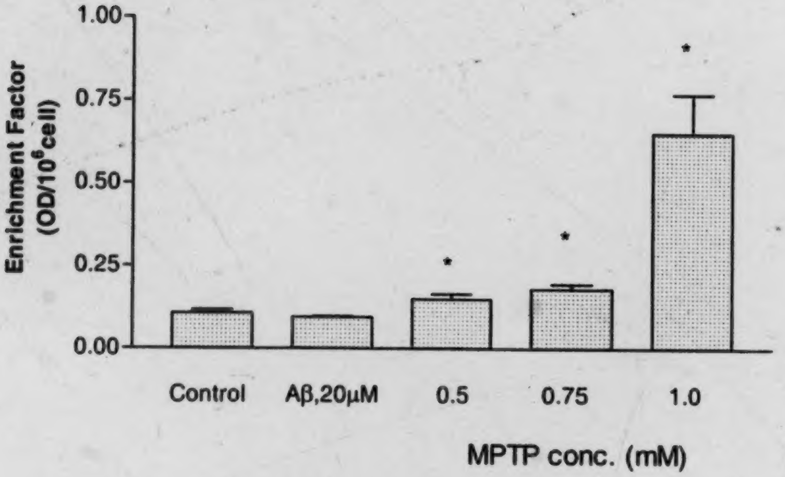


Fig. 3.13. ELISA quantification of mono- and oligo-nucleosomes in Aβ (20μM)- and MPTP (0.75mM)-treated SK-N-SH cells after 96hr of culture.

Cells were suspended at 3×10^4 cells/ml, with a total volume of 100μl /well in 96 well plate. Aβ and MPTP were added to the cell culture when initiating experiments. Apoptosis was investigated by using the ELISA method to quantify the mono- and oligo nucleosomes after 96hr.

*, $p<0.05$, when compared with the control group. (n=4)

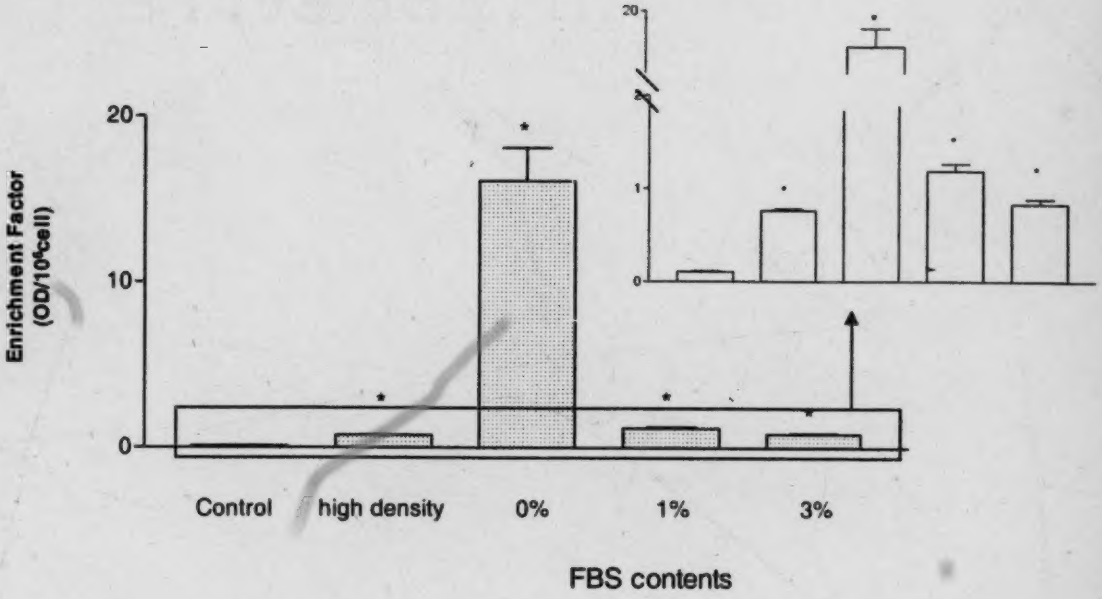
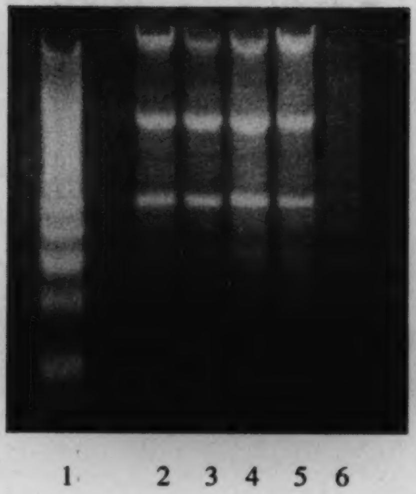


Fig. 3.14. ELISA quantification of mono- and oligo- nucleosomes in high density- and serum deprivation-treated SK-N-SH cells after 96hr of culture.

Cells were suspended at 3×10^4 cells/ml, with a total volume of 100μl /well in 96 well plate. Apoptosis was investigated by using the ELISA method to quantify the mono- and oligo nucleosomes after 96hr in high density culture and serum deprivation models.

*, $p<0.05$, when compared with the control group. (n=4)



1. Positive control

2. control
3. Aβ
4. MPTP
5. High density
6. FBS(-)

Fig. 3.15. The detection of apoptosis in multiple insult-treated SK-N-SH cells after 96hr of culture using the DNA laddering method.

Cells were harvested after being incubated with each insult for 96hr, and DNA was isolated. When apoptosis occurs, a ladder pattern shows on agarose gels after eletrophrosis.

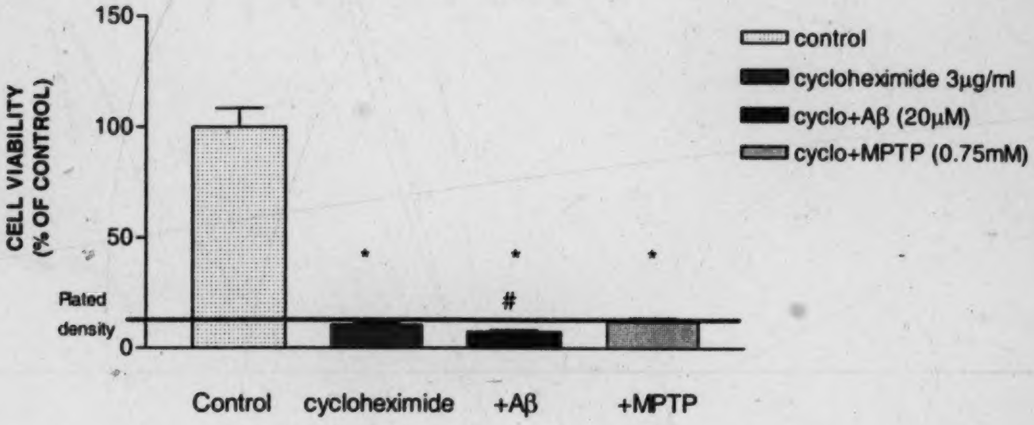


Fig. 3.16. The effects of Aβ (20μM) and MPTP (0.75mM) on cycloheximide (3μg/ml)-treated SK-N-SH cells in 96hr culture.

Cells were suspended at 3×10^4 cells/ml, with a total volume of 100μl /well in 96 well plate. Cycloheximide blocked proliferation by inhibiting protein synthesis. The effects of Aβ and MPTP were tested in SK-N-SH cells when protein synthesis was inhibited. Cell viability was assayed after 96hr. (Horizontal line indicates the cell density when initiating experiments)

*, $p<0.05$, when compared with the control group; #, $p<0.05$, when compared with the cycloheximide treated group. (n=3)

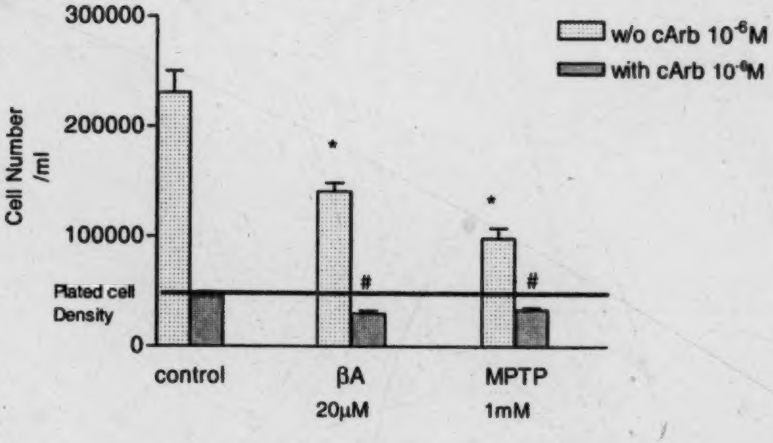


Fig. 3.17. The effects of Aβ (20μM) and MPTP (0.75mM) on cytosine-arabinoside (1μM)-treated SK-N-SH cells in 96hr culture.

Cells were suspended at 3×10^4 cells/ml, with a total volume of 100μl /well in 96 well plate. cArb blocked proliferation by inhibiting DNA synthesis. The effects of Aβ and MPTP were tested in SK-N-SH cells when cell prliferation was inhibited. Cell viability was assayed after 96hr. (Horizontal line indicates the cell density when initiating experiments)

*, $p<0.05$, when compared with the control group; #, $p<0.05$, when compared with the cycloheximide treated group. (n=3)

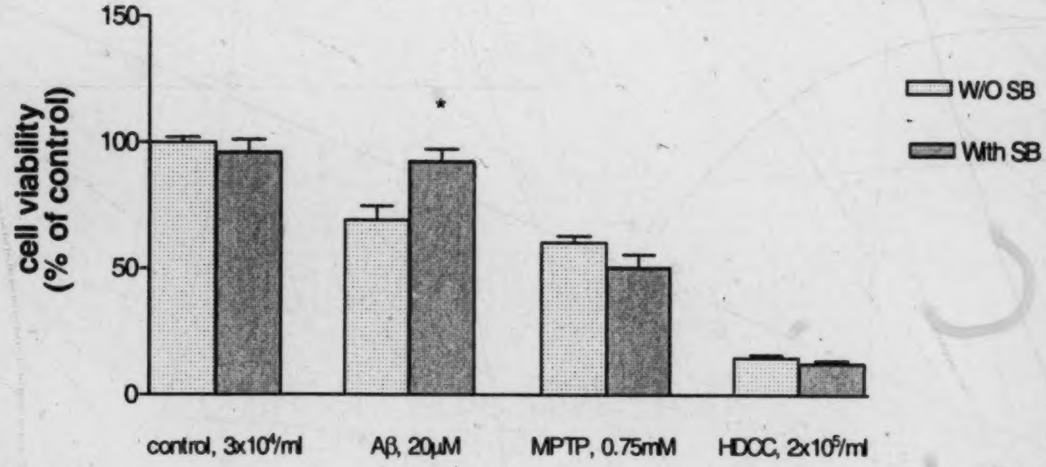


Fig. 3.18. The effects of the MAP kinase inhibitor, SB203580 on Aβ (20μM)- and MPTP (0.75mM)- and high density culture-treated SK-N-SH cells in 96hr culture.

Cells were suspended at 3×10^4 cells/ml, with a total volume of 100μl /well in 96 well plate. SB203580 (1μM) was added to Aβ, MPTP, and high density treated cultures. Cell viability was assayed after 96hr.

*, $p < 0.05$, when compared with the relevant Aβ-, but non-SB203580-treated group. (n=4)

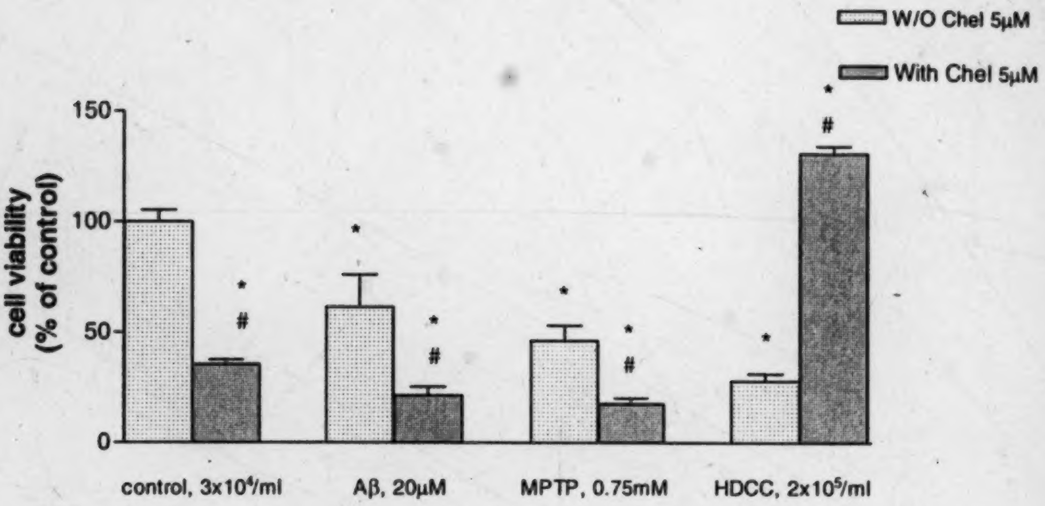


Fig. 3.19. The effects of PKC inhibitor, chelerythrine (5μM) on Aβ (20μM)-, MPTP (0.75mM)- and high density-treated SK-N-SH cells in 96hr culture.

Cells were suspended at $3x10^4$ cells/ml, with a total volume of 100μl /well in 96 well plate. Celerythrine was added to Aβ, MPTP, and high density treated cultures. Cell viability was assayed after 96hr.

*, $p<0.05$, when compared with the control group; #, $p<0.05$, when compared with the relevant non-celerythrine treated group. (n=4)

Chapter 4

The Neuroprotective Effects of Estrogen in SK-N-SH Cell Culture

4.1. Introduction

4.1.1. The many aspects of estrogen as a neuroprotectant

A woman spends about one-third of her life in her postmenopausal years in a hormonaly deficient state. [451] Research has demonstrated that postmenopausal women, as well as ovariectomized women, with low blood estrogen concentration, undergo multi-organ dysfunction, including bone loss, increased risk of coronary diseases, and mood, mental state and memory changes. [605] Estrogen replacement therapy (ERT) has been demonstrated to improve the above symptoms to a certain degree. Recent research has shown that ERT in postmenopausal women protects or delays the occurrence of AD, and improves memory loss caused by aging. [41, 518, 519]

It has been suggested that estrogen exists in several areas of the brain involved in emotion, memory and sexuality. [194, 379, 519, 605] Estrogen acts on both genomic and non-genomic mechanisms that cause changes in the level of neurotransmitters and their synthesizing enzymes. Our original understanding of the role of estrogen in the CNS is derived from studies on the action of the hormone on modulating sexual behavior and differentiation. Several lines of evidence suggest that the effects of estrogen reflect a direct action of the hormone on neurons. The neuronal elements within the CNS that are responsive to the hormone are selectively endowed with the ER. The viability of *in vitro* cultures of differentiated hypothalamic neurons was prolonged by the addition of estrogen. [78, 216, 462] In developing brain explants and PC12 cells, estrogen stimulated neurite outgrowth. [437, 561] This effect of estrogen is similar to that of the neurotrophins that induce mechanisms necessary for neuronal survival and functions related to transmitter production and release. In addition, in cholingergic neurons of rodents and primates, receptors for NGF and the receptors for estrogen are both expressed and functional within the same cell, and the expression of the NGF receptors is modulated by estrogen. [194, 404, 560] Furthermore, the estrogen response elements have been demonstrated in the promoter region of the NGF gene and in the genes of other neurotrophins, including BDNF and epidermal growth factor. [100, 446]

In investigations of clinical effects of estrogen as a neuroprotectant and a neuromodulator, a few groups have reported that in postmenopausal women, estrogen administration alters mood, [42, 604] and memory. [208, 210, 605] Reports have suggested that postmenopausal women with lower levels of endogenous estrogen may be predisposed to the development of AD. Using dehydroepiandrosterone (DHEA) as a marker for endogenous estrogen production, several studies reported lower levels of this estrogenic precursor in subjects with AD than in age-matched controls both with and without other dementia. [129, 337] Several small (from 7 to 15 subjects), nonrandomized, open-label trials of estrogen treatment of patients with AD have been reported. [60, 238, 420, 512, 583] These studies demonstrated significant improvement in several global measures of cognitive function compared with baseline in most of the patients presented. This provides promising evidence that estrogen could be a useful candidate for therapeutic use in degenerative diseases and brain injuries.

4.1.2. Estrogen Receptors: concepts, functions, and mechanisms

Estrogen is important in both men and women for a variety of physiological processes. Many of the effects of estrogens are mediated through binding to nuclear ERs, which are transcription factors that regulate expression of estrogen-responsive genes. Other natural compounds and synthetic drugs are also capable of binding to the ERs.

Some of these compounds mimic the effects of estrogen; others have more antiestrogenic activity.[129, 271]

Estrogen and other ligands influence gene expression and cellular phenotypic changes by diffusing into the cell and binding to ERs in the nucleus. This binding activates the dimerization of these receptors, which further facilitates the direct interaction of receptor dimers with promoter regions in the DNA of target genes to activate or repress transcription. The two isoforms of ER, ERα and β, are nuclear hormone receptors that function as transcription factors when they are bound by the ligands. The nuclear receptors share common structural and functional features; their functional domains have been designated A-F (Fig. 4.1).[449] The classical ER (ERα) contains 595 amino acids with a central DNA-binding domain (DBD), along with a carboxy-terminal hormone-binding domain (HBD). Recently, ERβ has been identified (Fig. 4.1).[449] ERβ is somewhat shorter than ERα, containing 530 amino acids. The region of highest homology between ERα and ERβ is in the DBD (95%). There is less conservation in the A/B, D, and E domains.[310]

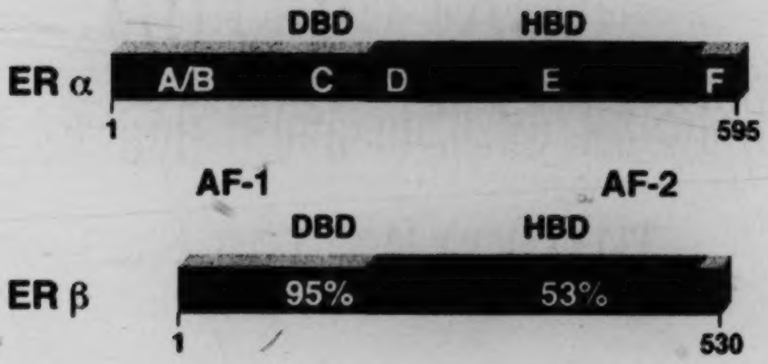

DBD
HBD
ER α
A/B
C
D
E
F
1
595
AF-1
AF-2
DBD
HBD
ER β
95%
53%
1
530

Fig. 4.1. The structures of ERα and β.

The structures of nuclear ERα and β. The functional domains are designated A-F. The 2 distinct transactivation domains AF-1 and AF-1 are located at A/B region and E region of the receptor, respectively. The DBD regions of ERα and β share high homologue, while the HBD regions are less conserved (modified from [449]).

The two ER subtypes may also have distinct physiological roles, as suggested by their different structures, especially in the crucial HBD where there is considerably less homology (53%). ER-mediated gene transcription is stimulated through at least two distinct transactivation domains located in the amino-terminal A/B region (AF-1) (also known as the immunogenic domain), and the carboxy-terminal E region, the HBD of the receptor (AF-2). [335, 471] Both AF-1 and AF-2 are required for maximal ER transcriptional activity, and most cells require both AF-1 and AF-2 functions for transcriptional activity when stimulated by estrogen. However, with certain promoters, AF-1 and AF-2 can function independently. Estrogen can stimulate transcription in some cells via receptors with only an active AF-1 or an active AF-2 function. Recently, it has been demonstrated that the activity of the ERβ AF-1 region is negligible compared with the AF-1 of ERα, but the transcriptional activity of AF-2 is comparable between the two. Thus, the activity of ERα may exceed that of ERβ on estrogen responsive element–containing genes that require both transactivation domains. It is also known that antiestrogens such as tamoxifen can function as pure antagonists on genes that require only the AF-2 domain for ER-mediated transcriptional activity. In contrast, in genes for which the ER AF-2 domain is not required, transcription is driven only by AF-1 and tamoxifen can function as a partial agonist. [116, 419] It is this capacity of many antiestrogens to act differently upon the two transactivation domains that, in part, makes them clinically useful antagonists in the breast but agonists in uterus, bone, and liver. This category of antiestrogens, which have many estrogenic effects depending on the tissue and gene, are more appropriately called selective estrogen receptor modulators (SERMs).

After hormone binding and dimerization, ERs bind to DNA with high affinity through their DBD at the estrogen responsive elements (Fig. 4.2). The binding of ER to other response elements in the promoter region of target genes may also be triggered by the binding of certain ligands. In addition, ERα and ERβ can form heterodimers that could also alter gene transactivation. [90] Besides this classical mechanism of direct DNA binding, the two ER subtypes can also activate other pathways. AP-1 response elements, important transcription factors, for instance, are regulated indirectly through interactions between ER and the AP-1 transcription factors *c-fos* and *c-jun*. [335] These transcription factors regulate genes involved in many cellular processes, including proliferation, differentiation, cell motility, and apoptosis.

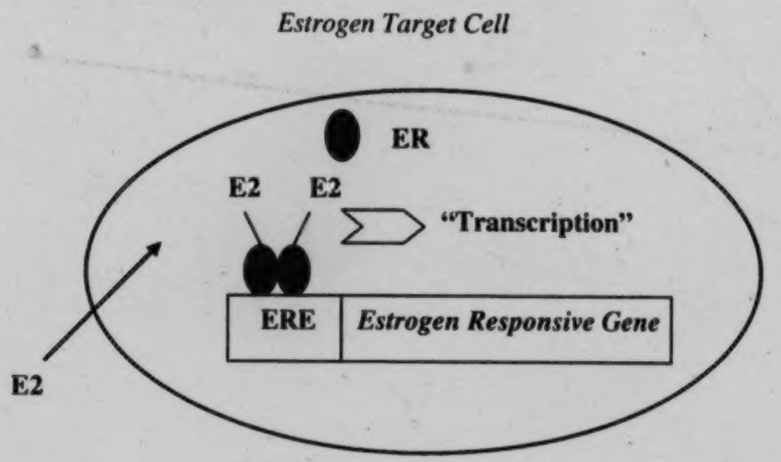


Fig. 4.2. The action of estrogen through ER- mediated pathway in estrogen target cells.

Estrogens such as estradiol (E_2) enter target cells by diffusion and bind the classic ER- (filled ovals). The receptor-hormone complex stimulates transcription of target genes via interactions with an estrogen response element (ERE) (adapted from [148]).

Cross-talk with other growth factor signaling pathways represents another way in which ERs can affect important cellular processes. For instance, members of the epidermal growth factor family of tyrosine kinase receptors can activate ER by directly phosphorylating crucial residues. [53] There is also considerable cross-talk between ERs and insulin-like growth factor (IGF) signal transduction pathways. The ER functions to increase levels of several of the key IGF signaling molecules, and IGFs, in turn, may activate ER. [146, 390] This cross-talk between signaling pathways that induce estrogenic effects in the absence of the hormone could conceivably contribute to the development of estrogen independence and/or clinical resistance to hormonal therapy.

The recognition that certain ligands can modulate the ER in different ways has led to an explosion in the development of new drugs tailored to have specific and selective effects on ER function. These drugs, of which tamoxifen is the prototype, are now collectively known as SERMs. Tamoxifen was developed more than 30 years ago and was approved for the treatment of advanced breast cancer. Its major metabolites are *N*-desmethyltamoxifen and *trans*-4-hydroxytamoxifen, which has an affinity for ER similar to that of E2. [359] Serum estrogen levels increase significantly in premenopausal women who take tamoxifen. Whether this limits its activity in such patients is not known.

Tamoxifen, like other SERMs, binds to the ER and, in breast cancer cells, antagonizes the effect of estrogen on a variety of growth-regulatory genes. The predominant effect of tamoxifen and many other SERMs is cytostatic with the induction of a G_1 cell cycle block, thereby slowing cell proliferation. In the CNS and vaginal mucosa, the predominant clinical effects of tamoxifen are also antiestrogenic. This accounts for the most common side effects attributed to tamoxifen, eg, menopausal symptoms. In most other tissues, tamoxifen predominantly exerts effects similar to those of estrogen. Tamoxifen is also estrogenic in bone, preserving bone density in postmenopausal women. However, bone density may fall in premenopausal women on tamoxifen, perhaps because it antagonizes the more potent activity of endogenous estrogen. [610]

A derivative of estradiol with a long, hydrophobic side chain at the 7α position, ICI 182,780, demonstrates a pure antiestrogenic profile on most genes and in most tissues studied to date. [291] ICI 182,780 binds to ERs (α or β) with an affinity similar to that of estradiol, and it exhibits 100 times greater binding affinity than tamoxifen. The mechanism of action of this steroidal antiestrogen differs significantly from other SERMs with mixed agonist/antagonist properties. In contrast to other SERMs, ICI 182,780 blocks ER transactivation coming from both the AF-1 and AF-2 domains. The drug may also impair ER dimerization, but most importantly, ICI 182,780 induces ER degradation, with a marked reduction in the cellular concentration of ERs. [291, 430] Because the ER is the major target for all antiestrogens, depletion of ER from the cell would theoretically constitute optimal therapy. As a consequence of these cellular effects, ICI 182,780 is a potent inhibitor of transcription of estrogen-regulated genes. In rat uterus, both estradiol and tamoxifen can stimulate expression of several genes, but in each case, ICI 182,780 has no activity on its own and it completely blocks estrogen or tamoxifen induction of these genes. In an *in vivo* model, ICI 182,780 was also much more potent than tamoxifen or estrogen withdrawal in blocking transcription of estrogen-regulated genes in human breast cancer cells. [247]

ICI 182,780 demonstrates promising antitumor activity in preclinical models. For instance, tamoxifen-resistant cell lines selected *in vitro* are sensitive to growth inhibition by ICI 182,780. [169] In many models, tumors become resistant to tamoxifen as a consequence of the development of tamoxifen-stimulated growth after an initial period of growth suppression. ICI 182,780 is a potent inhibitor of these tamoxifen-stimulated tumors, which suggests that it might have activity in patients who are resistant to tamoxifen, a prediction that has now been confirmed in the clinic. Serial biopsies from tumors in patients treated with either tamoxifen or ICI 182,780 show that the latter is a more potent inducer of apoptosis. [169] ICI 182,780 may not cross the blood-brain barrier and may, therefore, not cause hot flashes, a clinically important problem with other SERMs. [449] The cumulative data suggest that ICI 182,780 may be the most desirable SERM for both basic and clinical ER-related research.

The current studies investigate the neuroprotective actions of estrogen in multiple apoptotic or necrotic cytotoxicity models established with SK-N-SH cells. Whether the protective effects exerted by estrogen are mediated by ER activation are also studied.

4.2. Experimental design

4.2.1. The neuroprotective effects of estrogen

When cells were cultured in Aβ- or MPTP-containing medium, or under serum deprived or high density culture conditions, a significant decease in cell viability had been observed. To investigate the neuroprotective effects of estrogen against the cytotoxic insults, E2 was added to each of the above cytotoxic culture models when the experiments were initiated with concentrations ranging from 1pM to 0.1uM. The concentrations of E2 used in the current studies cover the range of all physiological relevant circulating levels of the hormone in a woman's body and the doses used in ERT in postmenopausal women. A concentration of E2 at 0.1μM was also chosen since other studies suggested an anti-oxidative action of E2 at similar concentrations. Cell viability was assayed after 96hr. A restoration of the cell viability by E2 in the above models would suggest a neuroprotective effect of estrogen.

4.2.2. The identification of the anti-cytotoxicity effect of E2

In normal culture conditions, cells undergo proliferation. Therefore, the apparent restoration of the cell viability by E2 could be through two possible mechanisms: promotion of proliferation, or protection from cytotoxicity. To define the mechanism of the neuroprotective effect, cArb (1μM) was used to block cellular proliferation. Thus, cell number would remain constant for the entire culture period. A further decrease in cell viability in any model would be a cytotoxic effect, and any protective effect to reverse that decrease would be an anti-cytotoxic effect or neuroprotective effect. Aβ (20 μM) or MPTP (1mM) were added to the culture, and the cytotoxicity was tested by assaying the cell viability after 96hr culture. One dose of E2 (0.1nM), as found to be optimal in the previous cytotoxicity study in the absence of cArb, was chosen to investigate the neuroprotective actions of estrogen in the presence of cArb.

4.2.3. ER antagonists and E2 effects

The effects of E2 observed after 96hr culture are likely to be genomic effects. Many of the effects of estrogen are mediated through the binding to ERs, and the estrogen-ER complex subsequently regulates expression of estrogen-responsive genes. To investigate whether the observed effects of E2 were through the ER-mediated pathways, tamoxifen and ICI 182,780 were added to the culture, and the effects of the antagonists on the actions of E2 were determined.

Tamoxifen, dissolved in DMSO as a 1mM solution, was diluted in MEM to a final concentration of 0.1, 0.3, 1, 3, 10, or 30μM. Thus, in the treatment groups with 10 and 30μM tamoxifen, 1% and 3% DMSO existed in the media, respectively. 1% and 3% DMSO were tested as vehicle controls in the culture. DMSO concentrations less than 1%, as would be seen with tamoxifen concentrations lower than 10μM, were not tested. ICI 182,780 was dissolved in DMSO as a 1μM solution, and then diluted in MEM to a final concentration of 10^{-6}M.

4.2.4. The expression of ER in SK-N-SH cells

To determine whether ERs were expressed in the SK-N-SH cell line, Western blot analysis was performed with anti-ERα monoclonal antibody and anti-ERβ polyclonal antibody (antibodies were produced by Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A, and were generously provided by Dr. Sandra Davidge), respectively. Protein preparations from rat myocytes which were previously identified to express both ERα and β (Dr. Sandra Davidge's observations, personal contact) were used as a positive control.

4.3. Results

4.3.1. The neuroprotective effects of E2

4.3.1.1. Aβ model

E2 (0.01nM ~ 10nM) provided significant protection against Aβ-induced cytotoxicity. Moreover, the optimal concentration of E2 (0.1nM), almost fully restored the decrease in cell viability at the concentration of Aβ used (Fig. 4.3). Concentrations higher than 0.1μM did not protect against Aβ toxicity in SK-N-SH cells, suggesting that the action of E2 was biphasic. The protective concentrations of E2 in the current study were in the range of circulating levels in premenopausal women (i.e. 0.01 ~ 0.1nM), and in women on postmenopausal ERT (1nM). [225] These data confirmed the results of Green *et al.* that 0.2nM of E2 significantly protected Aβ-induced live cell number decrease in the same cell line as determined by the trypan blue exclusion method. [225]

4.3.1.2. MPTP model

E2, in the same range of concentrations used in the Aβ model, significantly protected MPTP (0.75mM)-induced cytotoxicity (Fig. 4.4). E2 at 0.1nM was also the most effective in the MPTP model, reducing the MPTP toxicity by 50.22 ± 4.93 % ($p<0.01$, $n=4$). Compared to the effect of E2 in the Aβ model, which was almost a full restoration of cell viability, E2 did not show an protective effect in the MPTP model as effective as in the Aβ model.

4.3.1.3. Serum deprivation

In both the serum-free and 1% FBS models, E2 reversed the losses in cell viability. In the serum-free treatment group, all E2 concentrations (1pM ~ 10nM), showed protective effects (Fig. 4.5); in 1%FBS treated cells, the same range of E2 concentrations (0.01 ~ 10nM) restored cell number (Fig. 4.6). Bishop and Simpkins [63] reported that the addition of E2 (544pg/ml) to serum-free media significantly enhanced the number of live cells in SK-N-SH culture. Our results suggested that in both complete (serum-free) and partial depletion (1%FBS) of growth factor induced apoptotic neuronal death (see chapter 3), a relatively broad range of E2 concentrations exerted sufficiently protective effects, and that the effects of E2 were biphasic. However, in this trophic factor withdrawal model, E2 at all the concentrations tested was only partially protective.

4.3.1.4. High density

In this model, extensive cell death was detected after 96hr culture due to multiple factors that were unfavorable to the culture. E2 ($10^{-11\sim-8}$M) significantly reversed the cell viability loss, with the most effective concentration being 0.1nM (Fig. 4.7).

In all 4 cytotoxic models with SK-N-SH cells, physiologically relevant concentrations of E2 showed protective effects when applied to the culture with the exposure of the cells to different insults. The most effective concentration of E2 in all four models was 10^{-10}M, a concentration similar to the circulation level in premenopausal women. These data suggested that the ovarian steroid may play a fundamental role in the protection of neurons from a large variety of insults. Moreover, the protective effects of E2 (0.1nM, for example) was the most effective in the necrotic Aβ model among all models. In the other apoptotic models tested, E2 only showed partial restoration of the cell viability.

4.3.2. Distinguishing stimulation of proliferation from inhibition of cell death

The observed restoration of cell viability in the cytotoxic models by E2 could be through two major pathways: a trophic effect, namely promoting proliferation, or through a neuroprotective effect by inhibiting cell death. In serum-free and high density models, the cell numbers assayed after 96hr culture were lower than the initial cell density, indicating a cell death process occurred in these two models. To further characterize the cell death in Aβ and MPTP models, cArb was added to cell cultures to inhibit proliferation. cArb (1μM) maintained the cell number at the plated density after 96hr culture (Fig.4.8), and the addition of Aβ (20μM) and MPTP (0.75mM) decreased cell viability by 34.46 ± 5.14 %, and 26.27 ± 4.17 %, respectively compared with cArb treated SK-N-SH cells (Fig. 4.8). E2, 0.1nM significantly, but partially, protected cell death in the Aβ and MPTP groups in the presence of cArb (Fig.4.8). These results illustrated that the restoration effects of E2 in the cytotoxic models investigated were at least partially through the anti-cytotoxic effects, namely through neuroprotective action. E2 may have played a role in regulating the proliferation in these cells, as well. Further investigations concerning the mitogenic effects of E2 are required.

4.3.3. The expression of the ER in SK-N-SH cells

Using Western blot analysis with anti-ERα and β antibodies, ERβ was expressed in SK-N-SH cell lines. This expression was seen in all cytotoxic treatments (Fig. 4.9). However, ERα could not be detected with Western blot analysis, suggesting that either

ERα was not expressed in this cell line, or the expression was too low to be detected by the technique.

4.3.4. The effects of ER antagonists in E2 treated cytotoxic models

4.3.4.1. Tamoxifen

Tamoxifen (0.1 ~ 10μM) did not show any effects on control SK-N-SH cell culture (Fig. 4.10); neither did the 1% DMSO vehicle. However, 3% DMSO and 30μM tamoxifen had a strong toxic effect. The toxicity was due to the effect of 3% DMSO alone.

In the Aβ model, serum-free, and 1%FBS treated culture, tamoxifen, at all concentrations (0.1 ~ 10μM) did not diminish the protective effects of E2 (Fig. 4.11 ~ 4.13). In the MPTP model, tamoxifen (1, 3, and 10μM) partially inhibited the E2 effects after 96 hr culture (Fig. 4.14). In the high density treated cells, tamoxifen concentrations at 3μM and 10μM dramatically increased cell viability, regardless of the presence of E2; while the lower concentrations tested did not modify the effects of E2 (Fig. 4.15). Tamoxifen also acts as a PKC inhibitor at the concentrations which showed protective effects in the high density model. [242, 283, 440] As observed in previous experiments presented (see chapter 3), the PKC inhibitor chelerythrine protected cells in the high density-induced cell death model. Therefore, it is possible that this action of tamoxifen was mediated through inhibition of PKC. The observations that the ER antagonist tamoxifen did not change the protective effects in Aβ, serum-free and 1% FBS models suggested that in these models the protective action of E2 was not through ER pathways. Moreover, tamoxifen, a SERM, can either be an agonist or antagonist of E2 in different tissues, as reported in the literature. [146, 247] Tamoxifen may somewhat mimic E2 effects in these models, as well. In the MPTP model, the observed effects of tamoxifen which neutralized E2 protection could be either through the blockade of E2 through ERβ mediated pathways, or through other unknown mechanisms involving apoptosis or neurotransmitter activities.

4.3.4.2. ICI 182,780

Compared with other SERMS, such as tamoxifen and raloxifene, ICI 182,780 is considered to have an ERα-selective partial agonist/antagonist function, [598] but is a pure ERβ antagonist. [291, 430] ICI 182,780 (1μM) was added to SK-N-SH cells to further investigate whether the protective effects exerted by E2 in different cytotoxic models were acted through ER mediated pathways. Under normal culture conditions, when cells were exposed to both ICI 182,780 and E2 (0.1nM) together, an increased cell viability was seen, while neither E2 nor ICI 182, 789 alone had a mitogenic effect (Fig. 4.16). In both Aβ and high density models, ICI 178, 820 did not diminish the protective effects of E2 (Fig. 4.17, 4.18), suggesting that the protective effects of E2 in these 2 models were not through the ER mediated pathways. Interestingly, in the Aβ model, ICI 182,780, with or without E2, showed profound protective effects against Aβ (Fig. 4.17).

Among all the toxic groups, ICI 182,780 significantly blocked the effect of E2 in the MPTP model (Fig. 4.19), suggesting that the protective effect exerted by E2 in MPTP model was mediated by ERβ, and the protection could be blocked by the addition of the pure ERβ antagonist, ICI 182,780.

4.4. Discussion

In the current study, we have revealed that E2 had neuroprotective effects against neuronal death induced by multiple insults, involving anti-apoptotic and anti-necrotic mechanisms. The protection was through both ER mediated pathways (MPTP model) and non-ER mediated pathways (Aβ, high density, and serum deprivation models).

Estrogen has been observed to exert neuroprotective effects in various neurons of different species, [39, 45, 103, 294] and ERT in postmenopausal women has shown positive results in mood, cognition and memory. [156, 246, 451, 518, 519]

The current study has revealed that E2 acted as a neuroprotectant in neuronal cell death induced by several insults, including the Alzheimer's toxin, Aβ, the dopaminergic toxin, MPTP, serum deprivation or low serum culture, and a nutrient deprived high density model. E2 restored cell viability losses in both apoptotic and necrotic cell death, and in all four models, the physiological relevant concentrations of E2 was more effective. The properties of the 4 cytotoxic models, and the actions of E2 are briefly summarized in Table 4.1.

Table 4.1. Characterization of cell death in 4 cytotoxic models.

Models Observation	Aβ	MPTP	Serum deprivation	HDCC
Apoptosis	-	+	+++	++
MAPK inhibition	+	-	-	-
PKC inhibition	-	-	-	+
E2 effect	+++	++	+	+
Effects of ER Antagonists	-	+	-	-

Table. 4.1. Summary of the properties of the 4 cytotoxic models, and the effects of E2 on cytotoxicities, with or without ER antagonists. It has been observed that E2 protected neuronal cells from Aβ-induced necrosis, and MPTP-, serum deprivation-, and HDCC-induced apoptosis. Each cytotoxic model may involve a different signal transduction pathway, as revealed by the effects of different kinase inhibitors. Moreover, only in the MPTP model, not the others, the addition of ER antagonists abolished E2 effects, indicating that the protective action of E2 in the MPTP model was through ER-mediated pathways.

4.4.1. The neuroprotective actions of E2 against apoptosis and necrosis

4.4.1.1. Anti-Aβ-toxicity, an anti-necrotic action

The observations that physiologically relevant concentrations of E2 were capable of reversing the cell death induced by Aβ support the hypothesis that ERT is beneficial in AD. There are other reports demonstrating that estrogen has shown protective effects in Aβ-induced neuronal death, including the observations of Green and co-workers on SK-N-SH cells. [225, 232] These authors demonstrated for the first time that a concentration of E2 (0.2nM), which was within the physiologically circulating levels in premenupausal women, could attenuate the toxicity associated with exposure of SK-N-SH cells to a neurotoxic fragment of Aβ, Aβ 25~35. [225] Our findings confirmed what Green and colleagues have reported, and revealed that a relatively large range of concentrations of E2 was capable of providing neuroprotective effects against Aβ induced cytotoxicity. Among the concentrations of E2, physiological concentrations (0.1 ~1μM) tended to exert a greater degree of protecting, indicating that the dosage of E2 in ERT was sufficient to induce beneficial effects in elderly patients as reported previously. [62, 117, 246, 269, 452, 518, 519] Earlier reports by Behl *et al.* [45] and Goodman *et al.* [217] reported that E2 at μM concentrations protected primary cultured neurons from Aβ toxicity. Keller *et al.* [313] found that E2 at 10μM attenuated oxidative impairment of synaptic Na^+/K^+-ATPase activity, glucose transport, and glutamate transport induced by Aβ. The concentrations of E2 in the above studies were higher than the dosages for ERT and were also greater than those observed in pregnant women (50nM). The reported protective effects of E2 at pharmacological concentrations were more likely to be through its antioxidant activity (see more detail cascades of Aβ toxicity in chapter 1). The present observation regarding the efficacy of E2 at physiological concentrations against Aβ toxicity illustrated that E2 might play a fundamental role in the protection of neurons from various insults, including pathological changes associated with AD.

A role for estrogen in apoptosis regulation has been suggested in non-neural hormone-responsive tissues, such as breast [106, 551] and uterus. [366] Estrogen appears to modulate cellular viability by classical ER-dependent regulation of the anti-apoptotic protein *Bcl-2*. The well-established responsiveness of specific brain regions to estrogen suggests the possibility that estrogen may modulate neuronal vulnerability to apoptosis by acting as an endogenous regulator of apoptosis-related factors. Given the apparent involvement of apoptosis in AD neurodegeneration, the therapeutic benefits of ERT may reflect in part an inhibition of apoptotic pathways in estrogen-responsive neuronal populations, [465] with an involvement of $Bcl\text{-}_{XL}$, an anti-apoptotic member of the *Bcl* family closely related to *Bcl-2*. In addition, Gollapudi and Oblinger [212] reported that estrogen had clear cytoprotective effects on terminally NGF differentiated ER-transfected PC12 cells. The estrogen effects were synergistic with the effects of NGF in preventing apoptosis. Moreover, these cells treated with E2 during an apoptotic challenge showed alterations of important apoptosis-related gene products that might be involved in the neuroprotective effects of E2. However, with the observed cell death property in the current study with SK-N-SH cells, the protective effects of E2 in the Aβ model were likely through mechanisms other than inhibiting apoptosis, since Aβ did not induce apoptotic cell death as detected with oligonucleosome ELISA and DNA laddering methods. The observation that the optimal concentration of E2 (0.1nM) nearly fully reversed the toxicity of Aβ suggested that E2 was capable of inducing very strong and sufficient neuroprotection against necrosis or non-apoptotic pathways, as well.

4.4.1.2. E2, an anti-apoptotic agent in the MPTP model

SK-N-SH cells underwent apoptotic death with the exposure to 0.75mM MPTP after 96hr incubation. E2 (0.01 ~ 10nM) significantly protected against neuronal death, suggesting that E2 could be a potential candidate in the treatment of Parkinsonism. Several links between estrogen and dopaminergic neuronal degeneration have been reported. Sawada *et al.* [505] reported that preincubation with E2 provided significant neuroprotection against glutamate-induced neurotoxicity in rat mesencephalic dopaminergic neurons with an ED_{50} of 50μM. E2 also protected against dopaminergic neuronal death induced by $O_2.^-$ and H_2O_2 by suppression of ROS production. A more recent report [506] from Sawada's group indicated that E2 (10nM) provided neuroprotection against the apoptosis induced by buthionine sulfoximine and bleomycin sulfate in rat mesencephalic dopaminergic neurons, possibly by the anti-oxidant property of estradiol. In addition, a gender difference has been observed in the response to MPTP in mice. [402] MPTP caused a profound loss of dopaminergic neurons in the striatum of both male and female CD-1 mice. However, it was quite apparent that the depletion in the male was substantially larger than in the female (53% difference). The results suggested some aspect of female physiology provided neuroprotection against dopaminergic striatal damage induced by MPTP. In addition, our findings that physiological concentrations of

E2 prevented cell death induced by MPTP correlated with the above observations, and helped to explain the fact that PD occurrence is higher in men than in women. At the neurotransmitter level, E2 was able to attenuate the dopamine release induced by MPTP infusion into the corpus striatum. [149] These results implied that female hormonal modulation of nigrostriatal dopaminergic neurotoxicity might play an important role in the gender difference in PD.

The evidence concerning the protective effects of E2 in cell lines and tissue preparations other than primary nigral dopaminergic neurons was very limited. In one recent study of De Girolamo, [133] micromolar concentrations (10–1000 μM) of E2 were required to prevent MPTP-induced cell death. However, this was only achieved at concentrations which blocked cell differentiation in response to cAMP. This cytoprotective response could be a consequence of estradiol's anti-oxidative capacity or related to estradiol's ability to enhance a mitogenic/proliferative response within these cells. Our results were the first finding indicating that physiological concentrations of E2 were capable of protecting against the cell death induced by the dopaminergic toxin, MPTP, *in vitro* with neurons other than primary nigral dopaminergic neurons. This model has great potential for *in vitro* studies of PD related neuronal loss, and it is ideal for investigating potential neuroprotective candidates.

In addition, the observed protective effect of E2 was not a full reversal of cell viability loss in the current MPTP model. It suggested the anti-apoptotic action of E2 could only partially protect against MPTP induced cytotoxicity.

4.4.1.3. The effects of E2 on trophic factor withdrawal-induced apoptosis

Serum deprivation, a model that represents growth or trophic factor withdrawal, is a well established model in neuronal apoptosis research. It was found in the current study that growth/trophic factor withdrawal (serum-free, 1%, and 3% FBS in the culture) was a strong insult inducing neuronal apoptosis in SK-N-SH cells. E2, in a range of concentrations similar to those in the Aβ and MPTP models, significantly protected against the cytotoxicity caused by serum deprivation, suggesting an anti-apoptotic effect of E2 exerted in this model. The ability of E2 to protect SK-N-SH cells from serum deprivation has been reported previously. Bishop and Simpkins [63] reported that the addition of E2 (544pg/ml, approximately 2μM) to SK-N-SH cells in serum-free media maintained both total and live cell numbers at a level comparable to regular media at 24hr and 48hr. At 96hr of treatment with E2, cell number was more than twice the number observed in serum-free groups. The effects of estrogen against growth factor withdrawal have been reported in other cell preparations, as well. In rat hippocampal neuronal culture, the mRNA and protein of prostate apoptosis response-4 (Par-4), the product of a gene up-regulated in prostate cancer cells undergoing apoptosis, were elevated following the onset of trophic factor withdrawal. [101] E2 suppressed the induction of Par-4 production at the 12hr and 36hr time points. In our above observations, an anti-apoptotic phenomenon of E2 was illustrated. However, the precise mechanisms of the neuroprotective effect of E2 in the current serum deprivation model were unknown, but an E2-induced modulation of neurotrophins could not be ruled out. Toran-Allerand [560] has demonstrated the colocalization of ERs and NGF in the basal forebrain cholinergic neurons; in rats, ovariectomy reduced and E2 replacement enhanced NGF receptor mRNA in the cortex.[561] Also, intraventricular treatment with anti-NGF antibodies could antagonize some of the behavioral effects of neonatal E2 treatment. These data support the hypothesis that E2 can affect neuronal viability through induction of NGF receptors and/or NGF itself. In the SK-N-SH cell line, both the NGF receptor and its mRNA have been identified,[22] and E2 could affect the survival by modulating NGF receptors and NGF. However, a future study evaluating NGF receptor expression and activity by E2 is needed to prove the hypothesis.

As observed in the MPTP model, E2 only partially protected neuronal death in this trophic factor withdrawal model, suggesting that the anti-apoptotic actions of E2 cannot completely protect against cytotoxicity in the serum deprivation situation.

4.4.1.4. The effects of E2 on the high density model, a possible anti-ischemic action

The high density neuronal culture with SK-N-SH cells can be a useful *in vitro* model for investigation of brain ischemia. The similarities of this cytotoxic model to ischemia include nutrient depletion, oxygen and glucose deficit, accumulations of metabolic toxins, etc. The finding that circulating levels of E2 significantly inhibited cell number reduction in this high density culture supported the observations that the incidence of ischemic stroke was lower in premenopausal women compared to men.[154, 488, 584] The neuronal death in this model was defined as apoptosis by analyzing the cell death signal with oligonucleosome ELISA and DNA laddering gel. The anti-apoptotic properties of estrogen have been discussed in detail in the Introduction (Chapter 1). In brief, E2 can protect against neuronal apoptosis by stabilizing calcium homeostasis, reducing ROS, regulating caspase activities, maintaining mitochondrial membrane integrity, and increasing expression of anti-apoptotic proteins. It has been suggested that estrogen regulates *Bcl-2* expression in various tissues, including brain. [488] Dubal *et al.* [135] showed that E2 treatment in ischemic brain significantly increased *Bcl-2* levels and enhanced neuronal survival. Whether the protective effect of E2 in the high density model was through up-regulation of *Bcl-2* or other related anti-apoptotic proteins requires further investigation. Again, in the high density model, which is an apoptotic insult induced cytotoxic model, E2 was only partial by protective compared with an almost fully restoration of viability loss in the Aβ model. Therefore, it can be proposed that with SK-N-SH cells, E2 is more effective in protecting against necrotic neuronal death as in the Aβ model, and the effects of E2 were less profound against apoptotic insult-induced neuronal death.

4.4.2. The anti-cytotoxic property of E2

Among the 4 cytotoxic models, the serum deprivation and high density models severely reduced cell viability after 96hr culture, and the assayed cell numbers at the points of terminating the culture were even lower than the initially plated cell density. Therefore, the cell loss in these 2 models clearly reflected cytotoxic processes. A restoration of cell viability in these models could suggest a neural protective action. Thus, the observed effects of E2 were at least through anti-cytotoxic actions, namely neuroprotection. Bishop and Simpkins [63] in their serum-free study determined that after 48hr, E2 exposure did not increase thymidine uptake, indicating that the effects of E2 on SK-N-SH cells were cytoprotective rather than mitogetic. We have observed that in the presence of cArb (a DNA synthesis inhibitor), Aβ and MPTP further induced cell death when cell proliferation was inhibited, and the addition of E2 was capable of reversing the viability loss. This suggested that in all four models, the restoration of the cell viability by E2 was at least partially through the anti-cytotoxic effect. However, E2 could also have exerted mitogenic effects in some of the models which we had not defined. Usually, E2, at concentrations greater than μM, may exert mitogenic effects, [141, 524] as observed in tissues other than brain.

4.4.3. The protective action of E2, dependent or independent of ERs?

It has been postulated that estradiol may have neuroprotective functions at various cellular levels independent of the classical genomic action via its receptors. [562] These include intrinsic anti-oxidative properties [21] and the ability to enhance growth and differentiation of axons in developing brain through activation of the extracellular signal-regulated kinase pathway. [563] Our results have shown that neither tamoxifen or ICI 182,780 could abolish E2 effects on the Aβ induced cell death, trophic factor withdrawal or high density culture. Therefore, it can be postulated that for these models, after a 96hr culture period, cytosolic ER mechanisms were not the primary mechanism of the observed neuroprotection.

There has been strong evidence supporting mechanisms of neuroprotective effects of E2 independent of ER activation. Some *in vitro* studies have shown that high, non-physiological doses of estradiol were neuroprotective while low doses of estradiol, optimal to induce ER-mediated gene transcription, were not. For instance, 133 nM 17α-estradiol or E2, but not 1.3 nM E2, protected primary rat cortical neuronal cultures from anoxia-reoxygenation, or glutamate toxicity. [625] Both 17α-estradiol and E2 provided protection of SK-N-SH human neuroblastoma cells from serum deprivation and the neuroprotective effect was only slightly reversed by the ER antagonist tamoxifen. [224] Compared with its isomer, E2, 17α-estradiol is a much weaker ER agonist, and 17α-estradiol has a much lower affinity for ERβ (relative binding affinity of 17α- and E2 is 0.11:1).[506] In addition, 17α isomer is bound to the ER for a shorter duration than the 17β isomer. The protective effects of 17α-estradiol suggested a non-ER mediated pathway. Furthermore, protective effects of estradiol have been documented in neuronal cell lines lacking functional ERs or in the presence of ER antagonists. A neuroprotective effect of estradiol independent of ER has been demonstrated in the HT-22 murine neuronal cell line. 17α- Estradiol and E2, with an ED_{50} of 7nM and 5nM, respectively, protected HT-22 cells from Aβ toxicity, in spite of the fact that these cells lack functional ERs.[226] Neuroprotection independent of ERs has been observed in primary neuronal cultures as well. The ER antagonist, tamoxifen did not interfere with the neuroprotective effects of E2 against NMDA-induced neuronal death in rat hippocampal cultures.[297] Furthermore, neither ER antagonists nor protein synthesis inhibitors blocked estrogen-induced protection against glutamate neurotoxicity in mesencephalic cultures.[505] In addition, ER antagonists did not block the protective effects of estradiol against pro-oxidants in rodent and chick neuronal cultures.[21, 233, 313, 329] These findings suggested that estrogen might have neuroprotective effects that were not mediated by ER signaling, although another possibility was that tamoxifen exerted partial agonist activity in these cells. One of the most universally accepted explanations of the neuroprotective effects of the hormone concerns the endogenous antioxidant capacity of the estradiol molecule. It has been very well documented that estradiol has antioxidant properties and suppresses oxidative stress in neurons and neuronal cell lines induced by hydrogen peroxide, superoxide anions and other pro-oxidants.[42, 44, 45, 217, 413, 429, 461, 488, 496, 507, 515] The antioxidant activity of estrogens was observed to be dependent on the presence of the hydroxyl group in the C3 position on the A ring of the steroid molecule and was independent of an activation of the ER. [44, 329, 412] Some researchers found that E2 decreases iron-induced lipid peroxidation in rat brain homogenates, [575] rat cortical synaptosomes, [313] hippocampal HT 22 cells and primary neocortical cultures. [575] In addition to anti-oxidant effects, estradiol may have other possible neuroprotective mechanisms that are independent of nuclear ER activation. Estradiol may interact with estrogen binding sites in the plasma membrane [348] and may have many different rapid effects on neuronal excitability [417] and neuronal transmission. Neuroprotective effects that involve hormonal activation of intracellular signaling pathways via G proteins, [417] extracellularly regulated kinases, phosphorylation of the cAMP response element binding protein, [418, 464, 609, 625] and alterations in intracellular calcium levels are all possible mechanisms.

In the current study, the neuroprotective actions of estrogen in Aβ, serum deprivation and high density models were through non-ER mediated pathways, and the mechanisms probably involved its antioxidant property, since Aβ cytotoxicity has been closely correlated with oxidative damage (also see chapter 1, Introduction). [385, 405, 513, 530] The trophic factor withdrawal or brain ischemia induced apoptotic neuronal death correlates with mitochondrial dysfunction and free radical accumulation. Further studies on oxidative injuries in these toxic models may help to address how E2 modulates the antioxidant signal transduction pathways.

As detected by Western blot analysis, ERβ is expressed in SK-N-SH cells. The observation that ICI 182,780 antagonized the protective effects of E2 in the MPTP model suggested that the E2 protection in this model was through the ERβ mediated pathway. Many, though not all, of the physiological effects of estrogen are mediated through binding to ER proteins. Several lines of evidence suggested that neuroprotective effects of estradiol might at least in part be mediated by the activation of ERs. Direct proof of the involvement of ER on cell survival was obtained in PC12 cells. Estrogen enhanced the survival of PC12 cells transfected with full-length rat ERα, but did not affect the survival of control cells transfected with vector DNA alone. [213] There has also been evidence that ERs were involved in the regulation of neuronal survival in primary cultures. Estrogen enhancement of neuronal survival in primary hypothalamic cultures in the serum-free medium and in cortical cultures exposed to glutamate was blocked by the ER antagonists tamoxifen and ICI 182, 780. In addition, antiestrogens abolished the neuroprotective action of E2 in cultures of dorsal root ganglion neurons deprived of nerve growth factor. [194] All these findings provided evidence that estrogen may promote neuronal survival *in vitro* by an effect mediated via ERs. The dependence of neuroprotective effects of estradiol on ERs has been demonstrated *in vivo* as well. The intracerebroventricular administration of the ER antagonist ICI 182, 780 inhibited the neuroprotective effect of estradiol in hippocampal hilar neurons of ovariectomized rats exposed to systemic kainic acid. [194]

Moreover, there have been more interesting observations with the addition of the pure ERβ-antagonist, ICI 182,780. In the Aβ model, ICI 182,780, with or without E2, showed profound protective effects against Aβ (Fig. 4.19). Together with the observation that ICI 182,780 enhanced the mitogenic effect of E2 on SK-N-SH cells under normal culture conditions (Fig. 4.18), it is possible that the effects of ICI 182,780 on the Aβ model proceeded, at least partially, through the promotion of proliferation. Although ICI 182,780 is denoted as a pure ERβ antagonist, some studies have reported an agonistic potency that could be based on an ER subtype selective interaction. [572] The agonistic property of ICI 182,780 might have mediated a mitogenic effect in the above models. Moreover, ICI 182,780 has an ERα-selective partial agonist/antagonist function, but a pure antagonistic effect through ERβ. It may act to inhibit ERβ function, and subsequently change the balance of the expression between ERα and ERβ to favor the overexpression of ERα. The overexpression of ERα then might mediate a mitogenic effect in SK-N-SH cells. However, ERα expression could not be detected by Western blot analysis, suggesting that the basal expression was below the limit of detection. Future work to address regulation at the balance between ERα and ERβ may help to answer these questions.

4.5. Summary

The current study has found that E2 protected SK-N-SH cells against apoptosis or necrosis induced by different cytotoxic insults. The protective action of E2 is at various cellular levels and is mediated through various pathways. With the investigated neurocytotoxic system, the Aβ model involves neurotic cell death, while the MPTP, serum deprivation, and high density models induce apoptosis after 96hr culture. E2, at physiological relevant concentrations, was protective against all insults. The protective action in the MPTP model is the first finding indicating that physiological concentrations of E2 were capable of protecting against the cell death induced by the dopaminergic toxin, MPTP, *in vitro* with neurons other than primary nigral dopaminergic neurons. The present data provide *in vitro* evidence that different mechanisms are involved in the neuroprotective efficacy of estrogens depending on whether the injury model used is related to the apoptotic or necrotic type of neuronal cell death. Moreover, insult-specific differences in neuroprotection by E2 were observed. In Aβ induced cytotoxicity, E2 showed promising protective action, and almost fully restored the cell viability loss after 96hr. In neuronal apoptotic cell death as induced by MPTP, serum deprivation, and high density, the neuroprotection was only achieved partially. These findings suggest that the protective efficacy of E2, within the SK-N-SH cell line, is much stronger in necrotic neuronal death than that of apoptosis.

The neuroprotective actions were determined to be mediated by ERβ in the MPTP model, and were apparently independent of ERs in the other cytotoxic models. These findings indicate that prevention of MPTP induced apoptotic neuronal cell death is probably associated with transcriptional regulation, mediated by activation of ER. However, the fact that the protection of E2 in the other two apoptotic insults induced cell death was not diminished by the addition of ER antagonists suggest that the ER-dependency in the MPTP model is not mediated through an universal anti-apoptotic cascade. The independence of the neuroprotective effect of estrogen in excitotoxicity and other insults from ER-mediated pathways or protein synthesis has been demonstrated. Various mechanisms of the non-ER-mediated actions have been proposed, including antioxidant properties of estrogens, [21, 42] direct inhibition of NMDA receptors, [201] rapid release of calcium from intracellular stores ,[51] blockade of calcium entry via L-type calcium channels, [399] and stabilization of mitochondrial function. [385, 582] The present studies have shown that E2 can exert protective effects dependent or independent of the activation of ER pathways, and the dependency of ER is injury type- or insult-dependent, regardless of apoptotic or necrotic type of cell death. These observations are the first clear demonstrations that the ER-dependency in the neuroprotective actions may even vary within one cell line, and whether ER activation is required is determined by the injury type, rather than the cell death type.

The expression of the ERα was barely detectable in the cultured SK-N-SH cells. Therefore, the ER-mediated neuroprotection in the MPTP model was through the activation of ERβ. This indicated that ERβ mediated pathways are capable of exerting effective neuroprotection against certain apoptotic insults. Moreover, the interesting findings that the addition of ICI 182,780 changed the pattern of E2 effects on the Aβ model, and even under normal culture conditions imply a possibility that by fully antagonizing the ERβ, ERα could be overexpressed. It is hypothesized that the elevated expression of ERα may mediate the mitogenic effect of E2 as seen in normal culture and Aβ treated cultures (Fig. 4.18, 4.19). However, data are lacking in the current studies to verify this notion. Future studies are required to address the question.

The observations that E2 protected neurons from Aβ and MPTP induced neuronal cytotoxicity hold great promise for improving the clinical management of selective neurodegenerative diseases. The effects of E2 on the high density model imply a treatment strategy in brain ischemia. The fact that physiologically relevant concentrations of E2 have shown profound protective effects in all four models suggests that the circulating level of estrogen in premenopausal women might play a fundamental role in maintaining a large array of neuronal functions. Moreover, the detected concentrations of E2 used in the current study help to predict and exploit the potential therepeutically beneficial effects of estrogen in the CNS, especially in neurodegenerative and injury related brain disorders.

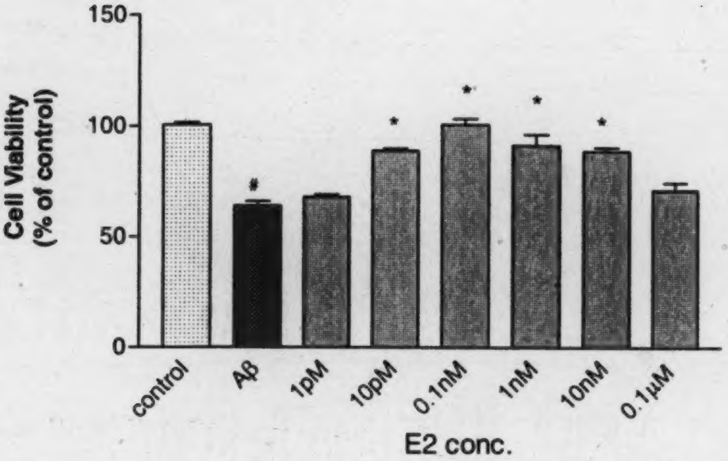


Fig. 4.3. The effects of E2 on Aβ (20μM)-treated SK-N-SH cells.

SK-N-SH cells were exposed to Aβ (20μM) for 96hr, with or without the addition of E2 (1pM ~ 0.1μM). Cell viability was assayed after 96hr of culture with WST-1.

#, $p<0.05$, compared with the control group;
*, $p<0.05$, compared with Aβ group. (n=5)

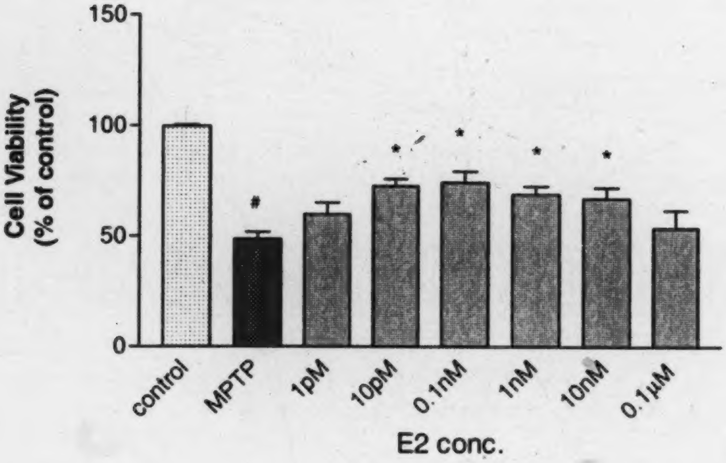


Fig. 4.4. The effects of E2 on MPTP (0.75mM)-treated SK-N-SH cells.

SK-N-SH cells were exposed to MPTP (0.75mM) for 96hr, with or without the addition of E2 (1pM ~ 0.1μM). Cell viability was assayed after 96hr of culture with WST-1.

#, $p<0.05$, compared with the control group;
*, $p<0.05$, compared with MPTP group. (n=4)

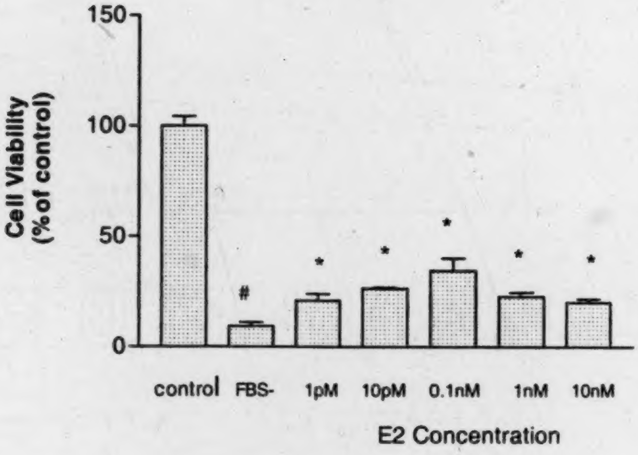


Fig. 4.5. The effects of E2 on serum (FBS) free-treated SK-N-SH cells.

SK-N-SH cells were exposed to serum-free media for 96hr, with or without the addition of E2 (1pM ~ 0.01μM). Cell viability was assayed after 96hr of culture with WST-1.

#, $p<0.05$, compared with the control group;
*, $p<0.05$, compared with FBS(-) group. (n=3).

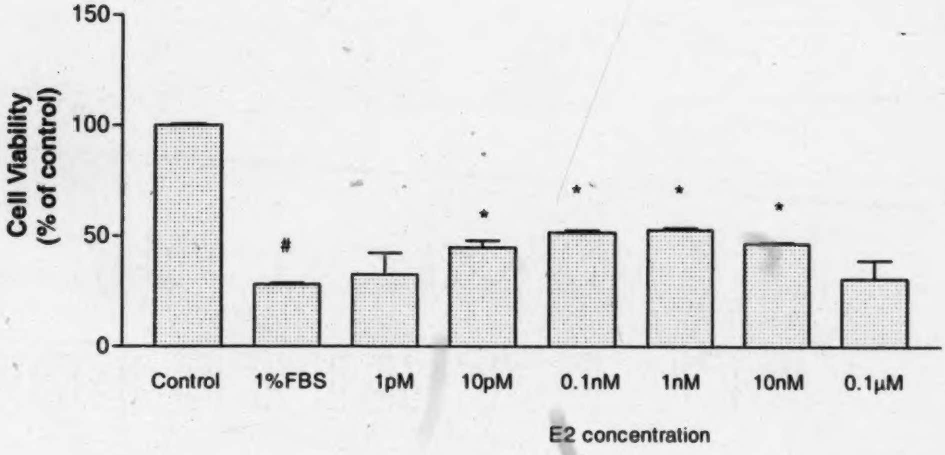


Fig. 4.6. The effects of E2 on 1%FBS media-treated SK-N-SH cells.

SK-N-SH cells were exposed to serum 1% FBS media for 96hr, with or without the addition of E2 (1pM ~ 0.1μM). Cell viability was assayed after 96hr of culture with WST-1.

#, $p<0.05$, compared with the control group;
*, $p<0.05$, compared with 1% FBS group. (n=5).

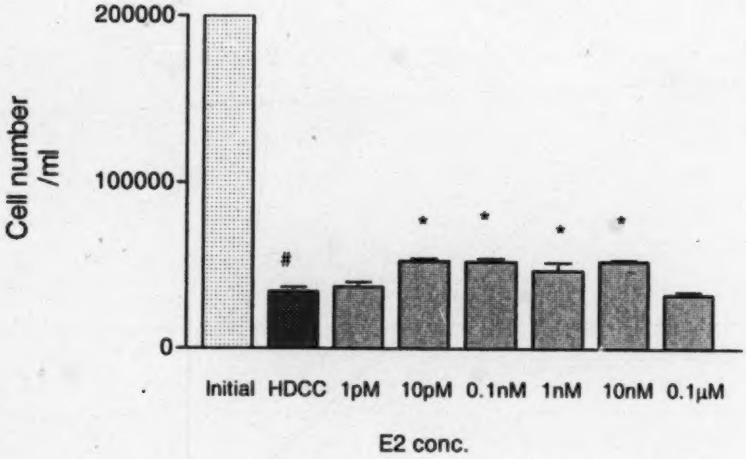


Fig. 4.7. The effects of E2 on high density culture-treated SK-N-SH cells.

SK-N-SH cells were plated at the density of 2×10^5 cells/ml for high density groups when initiating experiments, and cultures were maintained for 96hr, with or without the addition of E2 (1pM ~ 0.1μM). Cell viability was assayed after 96hr of culture with WST-1.

#, $p<0.05$, compared with the initial cell density;
*, $p<0.05$, compared with high density culture group. (n=5).

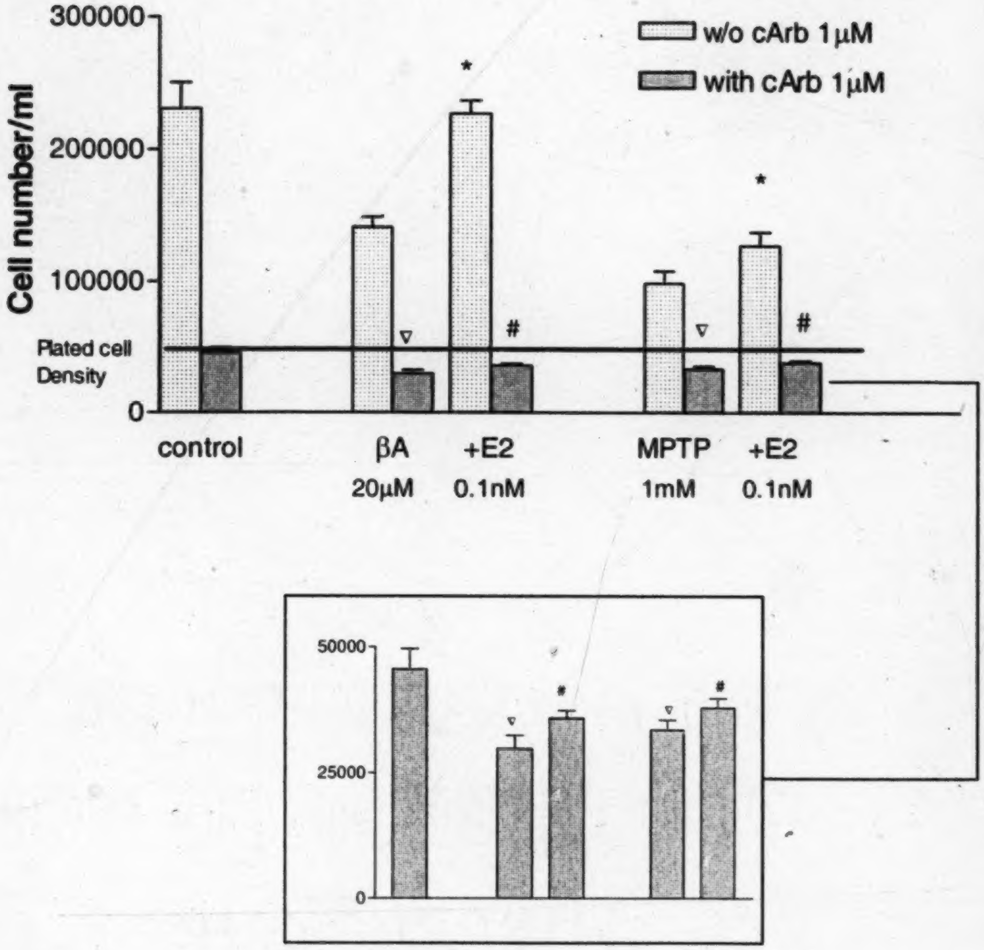


Fig. 4.8. The effects of E2 on Aβ- and MPTP-treated SK-N-SH cells with or without the addition of cytosine-arabinoside (1μM).

SK-N-SH cells were plated at a density of 5×10^4 cells/ml when initiating experiments, and cultures were maintained for 96hr. The effects of E2 (0.1nM) on Aβ (20μM) and MPTP (1mM) were investigated in the presence of cArb (1μM) which inhibited proliferation. Cell viability was assayed after 96hr of culture with WST-1.

*, $p<0.05$, compared with respective toxin treated groups;
∇, $p<0.05$, compared with cArb treated group;
#, $p<0.05$, compared with respective cArb treated toxin groups. (n=4).

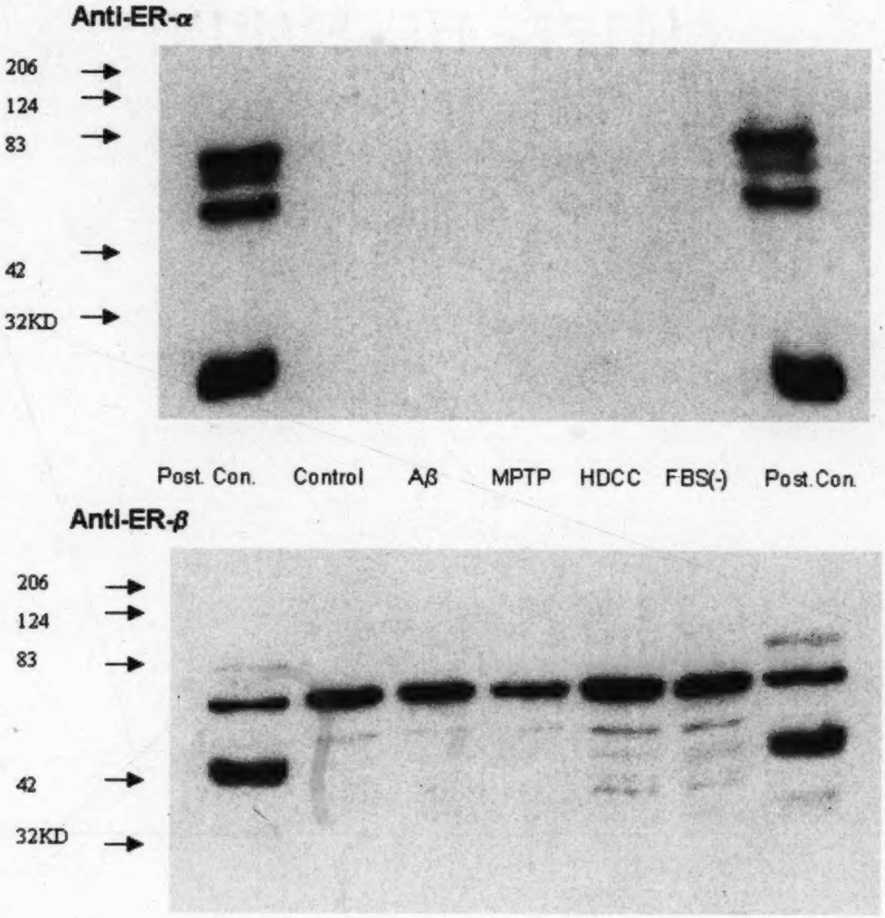

Anti-ER-α
206
124
83
42
32KD
Post. Con.
Control
Aβ
MPTP
HDCC
FBS(-)
Post.Con.
Anti-ER-β
206
124
83
42
32KD

Fig. 4.9. The detection of ERα and β in SK-N-SH cells.

The detection of ERα and β in SK-N-SH cells by Western blot with anti-ERα monoclonal antibody, anti-ERβ polyclonal antibody, respectively. The positive control in the experiment was a rat myocyte protein preparation which was previously identified to express both ERα and β. ERβ is expressed in SK-N-SH cells, while ERα cannot be detected with Western blotting analysis.

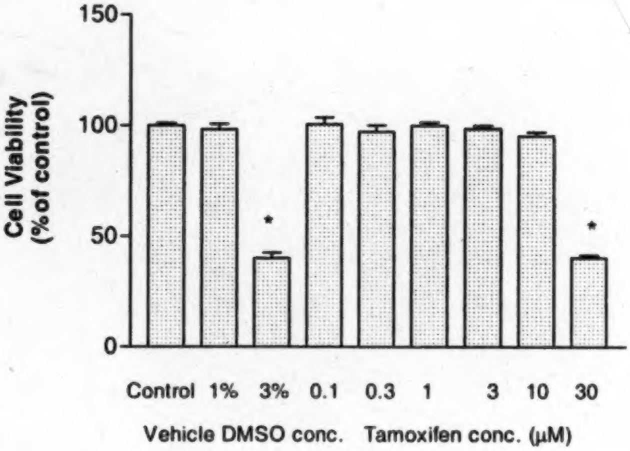


Fig. 4.10. The effects of tamoxifen on SK-N-SH cells.

SK-N-SH cells were cultured in the presence of tamoxifen (0.1, 0.3, 1, 3, 10, or 30μM). DMSO (1 and 3%) was also tested as the vehicle control. Cell viability was assayed after 96hr of culture with WST-1.

*, $p<0.05$, compared with the control group. (n=3).

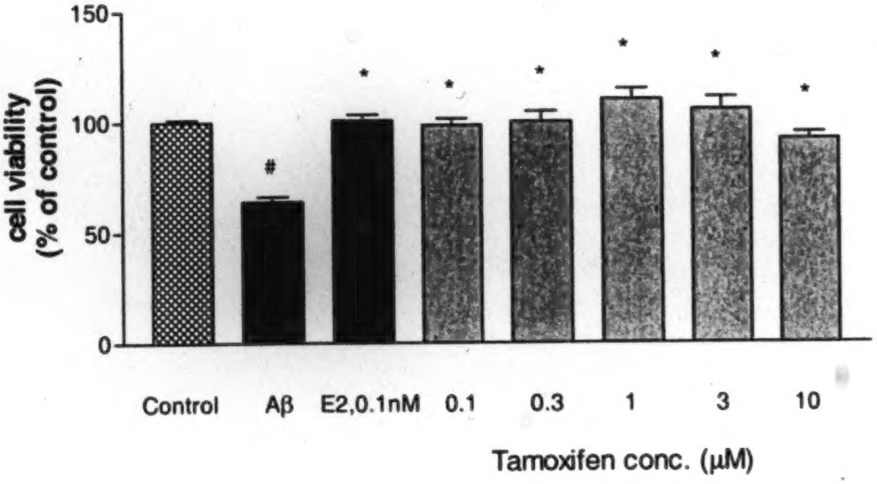


Fig. 4.11. The effects of tamoxifen on E2 (0.1nM)-protected Aβ(20μM)-treated SK-N-SH cells.

Tamoxifen, concentrations as indicated on the X-axis, was added to the culture which was in the presence of Aβ and 0.1nM E2. Cell viability was assayed after 96hr of culture with WST-1.

#, $p<0.05$, compared with the control group;
*, $p<0.05$, compared with the Aβ group. (n=4).

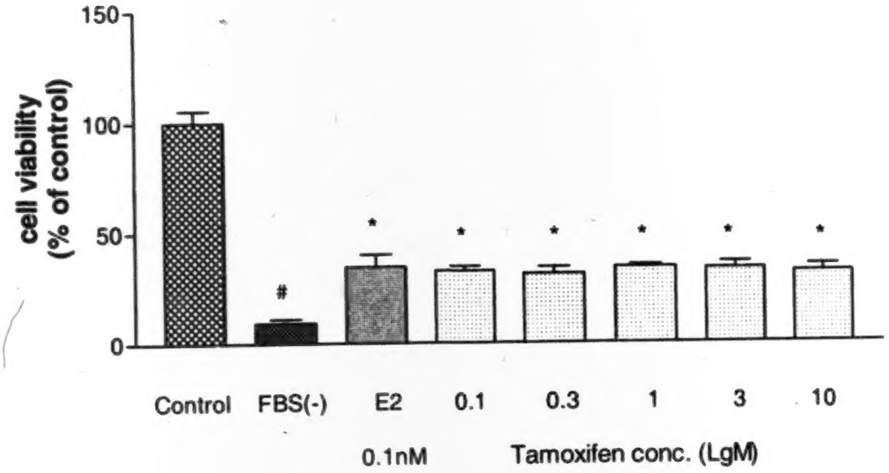


Fig. 4.12. The effects of tamoxifen on E2 (0.1nM)-protected serum deprived SK-N-SH cells.

Tamoxifen, concentrations as indicated on the X-axis, was added to the culture which was in the presence of FBS(-) media and 0.1nM E2. Cell viability was assayed after 96hr of culture with WST-1.

*, $p<0.05$, compared with the FBS(-) group;
#, $p<0.05$, compared FBS(-) with the control group. (n=4).

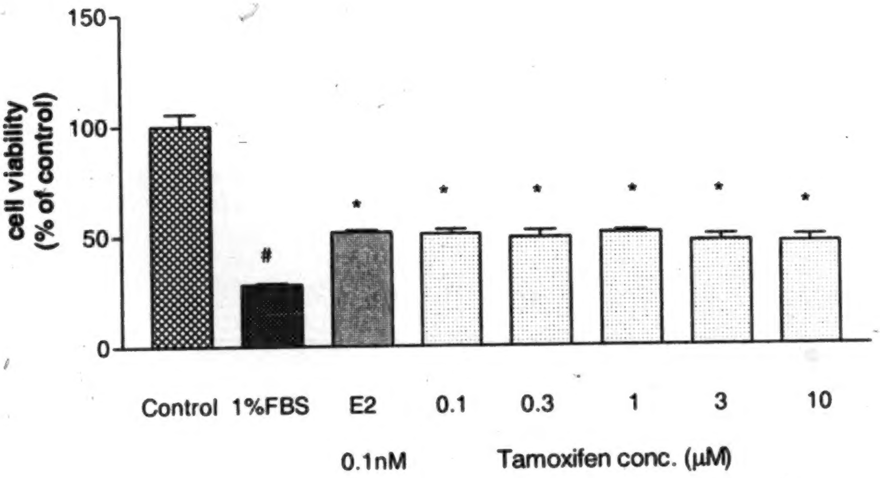


Fig. 4.13. The effects of tamoxifen on E2 (0.1nM)-protected 1%FBS media-treated SK-N-SH cells.

Tamoxifen, concentrations as indicated on the X-axis, was added to the culture which was in the presence of 1%FBS media and 0.1nM E2. Cell viability was assayed after 96hr of culture with WST-1.

*, $p<0.05$, compared with the 1%FBS group;
#, $p<0.05$, compared the 1%FBS group with the control group. (n=4).

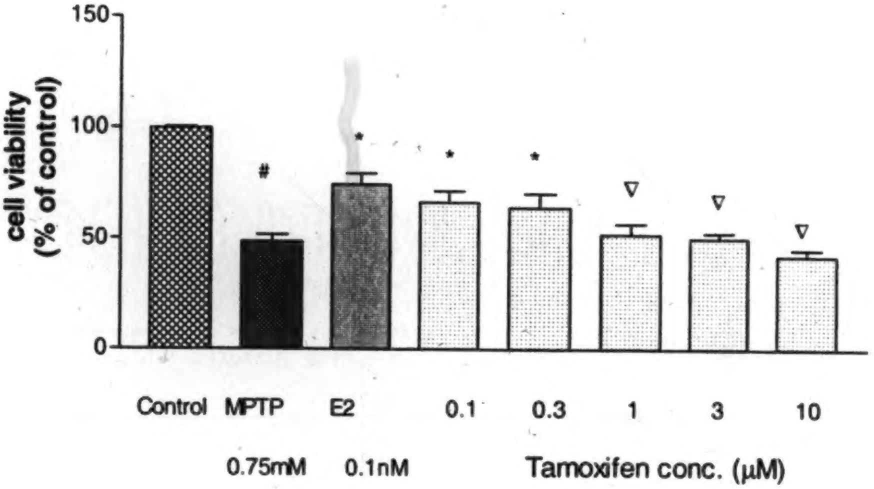


Fig. 4.14. The effects of tamoxifen on E2 (0.1nM)-protected MPTP (0.75mM)-treated SK-N-SH cells.

Tamoxifen, concentrations as indicated on the X-axis, was added to the culture which was in the presence of MPTP and 0.1nM E2. Cell viability was assayed after 96hr of culture with WST-1.

*, $p<0.05$, compared with the MPTP group;
#, $p<0.05$, compared MPTP group with control group;
∇, $p<0.05$, ∇∇, $p<0.01$, compared with MPTP+E2 group. (n=5)

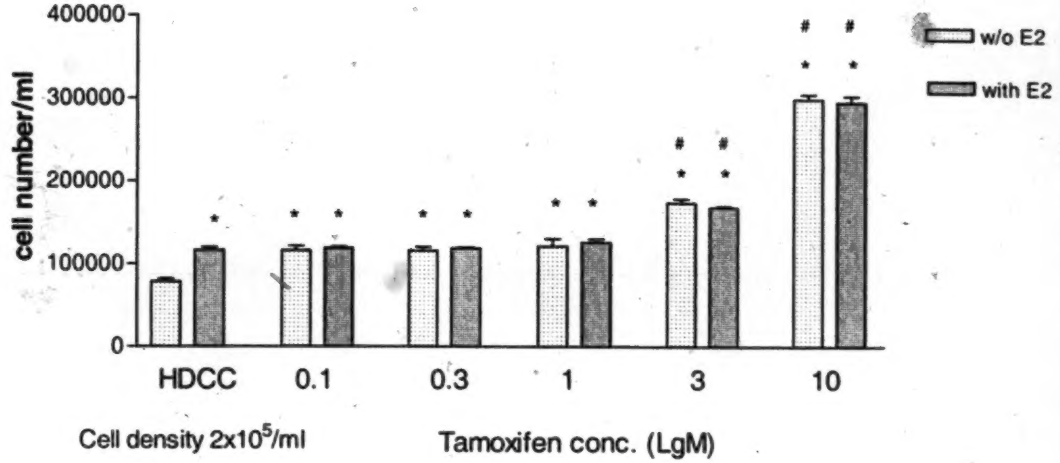


Fig. 4.15. The effects of tamoxifen on E2 (0.1nM)-protected high density-treated SK-N-SH cells.

Tamoxifen, concentrations as indicated on the X-axis, was added to the culture which was exposed to high density and 0.1nM E2. Cell viability was assayed after 96hr of culture with WST-1.

*, $p<0.05$, compared with the high density group;
#, $p<0.05$, compared the high density+E2 group. (n=5).

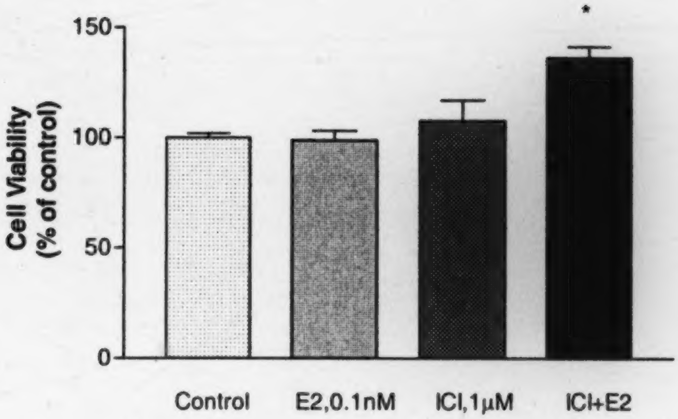


Fig. 4.16. The effects of ICI 182,780 on SK-N-SH cells.

ICI 182,780 (1μM) was added to the cell culture with or without the presence of E2 (0.1nM). Cell viability was assayed after 96hr of culture with WST-1.

*, $p<0.05$, compared with the control group. (n=4).

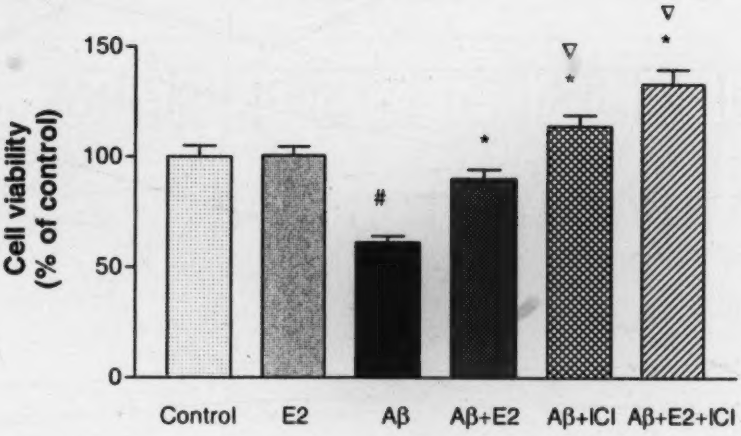


Fig. 4.17. The effects of ICI 182,780 on Aβ-treated SK-N-SH cells.

ICI 182,780 (1μM) was added to the cell culture, and the cells were exposed to Aβ (20μM), with or without the presence of E2 (0.1nM). Cell viability was assayed after 96hr of culture with WST-1.

#, $p<0.05$, compared with the control group;
*, $p<0.05$, compared with the Aβ group;
∇, $p<0.05$, ∇∇, $p<0.01$, compared with the Aβ+E2 group. (n=4).

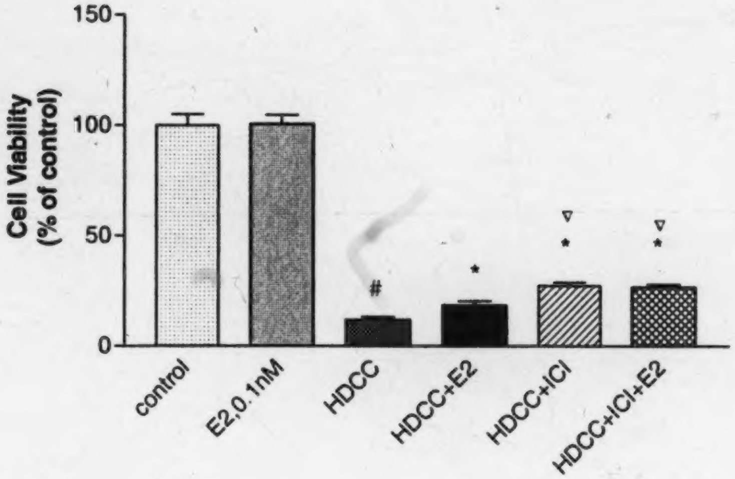


Fig. 4.18. The effects of ICI 182,780 on high density-treated SK-N-SH cells.

ICI 182,780 (1μM) was added to the cell culture, and the cells were exposed to high density, with or without the presence of E2 (0.1nM). Cell viability was assayed after 96hr of culture with WST-1.

#, $p<0.05$, compared with the control group;
*, $p<0.05$, compared with the high density group;
∇∇, $p<0.01$, compared with the high density + E2 group. (n=4).

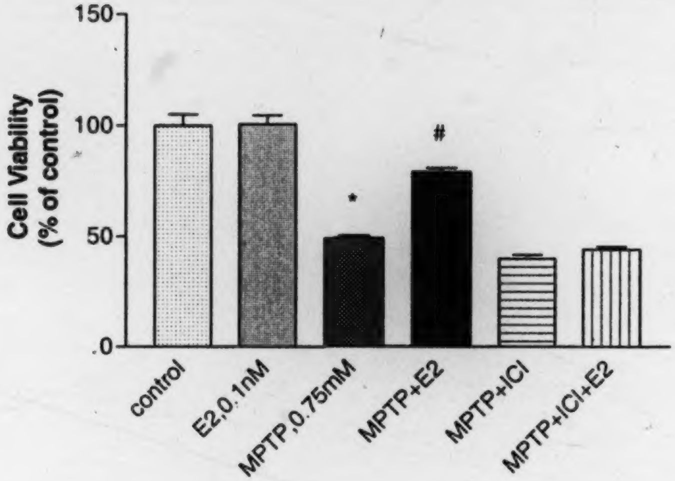


Fig. 4.19. The effects of ICI 182,780 on MPTP (0.75mM)-treated SK-N-SH cells.

ICI 182,780 (1μM) was added to the cell culture, and the cells were exposed to MPTP (0.75mM), with or without the presence of E2 (0.1nM). Cell viability was assayed after 96hr of culture with WST-1.

#, $p<0.05$, compared with the control group;
*, $p<0.05$, compared with the MPTP group. (n=4).

Chapter 5

The Role of Ca^{++} Channel Modulation in the Neuroprotective Actions of Estrogen in Aβ and MPTP Cytotoxic Models

5.1. Introduction

5.1.1. Voltage-dependent calcium channels and neuronal signaling

Intracellular Ca^{++} ion concentrations are pivotal to cell activation. Ca^{++} conductance in neurons has long been a focus of research in neurobiology due to the central role of this ion in the control of cell excitability and synaptic transmission. VDCCs are of particular functional importance in the regulation of calcium homeostasis, as they are involved both in electrical signaling itself, and in coupling electrical signals to changes in cytoplasmic calcium. The influx of Ca^{++} through neuronal VDCCs regulates a wide range of cellular processes, including neurotransmitter release, activation of Ca^{++}-dependent enzymes and second messenger cascades, gene regulation, and cell proliferation. [535]

Physiological and molecular studies have identified several different VDCCs. They are classified into low voltage-activated (LVA) channels, rapidly inactivating channels that are activated by weak depolarization, and high voltage-activated (HVA) channels, channels with variable inactivation that require stronger depolarization. [307] HVA channels are primarily responsible for most of the functions of calcium channels, including excitation-secretion and excitation-contraction coupling.

Much work has been devoted to classification of functional differences among HVA channels. The HVA channels of muscles are L-type, named in part for their long lasting activation and large single-channel conductance. In neurons, both L and N channels are usually expressed. The non-L HVA channels with diverse properties were defined as N channels. [167] N channels were then further defined as rapidly inactivating channels of intermediate single-channel conductance. The contemporary definition of L and T channels depends primarily on their pharmacological properties. L-type channels are highly sensitive to dihydropyridines (DHPs), and N channels are blocked by ω-conotoxin GVIA (CgTx). [567] Potentiation by DHP agonists is much more diagnostic for L currents than is inhibition by DHP antagonists. [439] CgTx usually inhibits mammalian N channels in an irreversible manner, but also produces a rapidly reversible block of some L channels. [600] P channels were originally identified in Purkinje neurons of the cerebellum, and are now defined by rapid blockade by ω-Aga IVA toxin. Q type channels were defined as DHP- and CgTx-resistant channels, and were blocked by ω-Aga IVA with lower affinity. [629] The distinction between P and Q channels is difficult to establish in many neurons. Therefore, many studies refer to P/Q channels unless high affinity blockade by ω-Aga IVA is unequivocal. P/Q channels are also blocked in a nearly irreversible manner by ω-conotoxin MVIIC. [629] Moreover, many neurons have a component of HVA current that is resistant to all of the above blockers (DHP, CgTx, ω-Aga IVA, and ω-conotoxin MVIIC). This resistant (R) current often inactivates more rapidly than other HVA currents, and may be activated at somewhat more negative voltage. [629]

In various preparations, the LVA T current consists of low threshold, fast inactivating, and slow deactivating channels. Unlike HVA, selective T-channel blockers are lacking. Posicor (mibefradil), developed by Roche, is the first and the only selective T-channel blocker. However, the product was withdrawn shortly after it was released. A few divalent ions also permeate Ca^{++} channels readily. Many others include the transition metals. Nickel, and also flunarizine, can block Ca^{++} channels, and are commonly used as non-selective T channel blockers.

The prototypical VDCC channel is composed of multiple subunits: the α_1 pore-forming units and other modulatory subunits, β, $\alpha 2$-δ, and γ (muscle). For both LVA and HVA Ca channels, one primary subunit (α_1) contains the domain necessary for basic channel function, including the calcium selective pore, the voltage sensor, and many other pharmacological characteristics [98, 99]. To date, the primary structures of at least nine different neuronal Ca^{++} channel α_1 subunits have been identified. L-type channels are composed of α_{1S}, α_{1C}, [600, 601] α_{1D} ,[557] and α_{1F}; [34] while α_{1B} forms N-type channels; [76, 158, 185, 600] α_{1A} is a component of both P- and Q-type channels; [77, 414, 502] α_{1G}, α_{1H}, and α_{1I} form T-type channels; [125] and α_{1E} probably is a component of the R current identified in several neuronal preparations. [534, 602] There are four internally homologous domains of the α_1 subunit, each containing six putative transmembrane segments (S1~S6). The S4 is highly unusual among transmembrane regions in that it contains several positively charged amino acids. The S4 segment is the voltage sensor that couples changes in membrane potential to channel opening. [165] An external loop between S5 and S6 is part of the ion conduction pathway, and plays a major role in calcium selectivity. [616] As expected from the functional differences, the LVA channel subunits are only distantly related to the HVA α_1 subunits, but crucial functional domains – the voltage sensors and pore loops - show strong conservation.

Neuronal VDCCs are multimeric complexes of at least three types of subunits as follows: α_1, $\alpha_{2\delta}$, and β. [635] L-type channels are encoded by four distinct genes, namely $Ca_v1.1$ to $Ca_v1.4$, [170] that give rise to numerous splice variants. Mammalian L-type channels have a similar ion selectivity and inactivation kinetics and are affected by DHPs at similar concentrations. The genes for four L-type calcium channels have been identified as follows: the skeletal muscle *$Ca_v1.1$* ($_{1S}$), the cardiac *$Ca_v1.2$* ($_{1C}$), the neuroendocrine *$Ca_v1.3$* ($_{1D}$), and the retinal *$Ca_v1.4$* ($_{1F}$) channel. Diversity within the α_1 subunit family is responsible for the major pharmacological and physiological features that distinguish the different classes of calcium channels. α_1 subunits contain the calcium pores and binding sites for selective channel antagonists. In the brain, α_{1C} and α_{1D} are localized to neuronal cell bodies and proximal dendrites.[268] Two splice variants of the rat α_{1C} subunit have been identified, α_{1C-1} and α_{1C-2}, and are differentially expressed in rat brain. The α_{1C-2} protein differs from α_{1C-1} by having a 3-amino acid (aa) insert in the cytoplasmic loop between domains II and III and a 28-aa substitution in the S3 segment in domain IV. In the human α_{1C} gene, this alternatively spliced IV-S3 transmembrane segment is encoded by homologous alternative exons 31 and 32 (23). It is not known if α_{1C-1} and α_{1C-2} differ in function.

VDCCs are both regulating and regulated. Calcium influx through VDCCs modulates cellular activities; cellular activities modulate calcium channels. This includes both long term modulation of channel expression and short term regulation of the activity of preexisting channels. In neurobiological research, the functions of cytosolic calcium as a second messenger which mediates a wide range of cellular responses are of great interest. Calcium influx into neurons acts as the critical transducer of electrical input into biochemical output. A wide range of neurotransmitter receptors and second messenger systems have been shown to regulate the influx of extracellular calcium through their effects on VDCCs and other ion channels by a process termed neuromodulation. However, the mechanisms by which calcium influx elicit long-term neuronal responses are less clear. Studies of the biochemical responses generated in response to calcium influx have focused on activation of cytoplasmic signaling molecules that directly bind calcium or calcium–complexes, such as calcium-CaM-dependent adenylate cyclases and protein kinases. [231] Calcium influx also leads, by indirect mechanisms, to activation of the ubiquitous MAP kinase pathway, [181, 489] which is a critical intermediate in long-term cellular responses such as proliferation and differentiation. [160] Greenberg's group have demonstrated that stimulation of the MAPK pathway in response to calcium influx through L-type VDCCs involves activation of the small guanine nucleotide binding protein Ras, [180] a protooncogene product that mediates MAPK activation in response to a wide variety of mitogens, cytokines, and trophic factors such as NGF. It has become increasingly apparent during the last decade that changes in $[Ca^{++}]_i$ can act more broadly to influence events such as neuronal survival, axon outgrowth, and changes in synaptic strength [207]. Moreover, depending on the mode of Ca^{++} entry, Ca^{++} can mediate disparate biological responses. For example, Ca^{++} entry through VDCCS can lead to neuronal survival in the embryonic CNS and peripheral nervous system, while Ca^{++} entry through NMDA receptors in postnatal neurons results in excitotoxic cell death. [207] Therefore, the mode of Ca^{++} entry and the condition of responding cells may be critical determinants for cell survival.

Within neurons, there are multiple Ca^{++} signaling pathways that are differentially activated by specific classes of Ca^{++} channels. In turn, activation of these distinct Ca^{++} signaling pathways can regulate the formation of transcription complexes and influence gene expression. A detailed review of Ca^{++} signaling pathways has been presented in the Introduction (Chapter1). On the other hand, the blockade of channels by the VDCC antagonists also modifies cellular function. For instance, the L-type blockers are widely used for treatment of hypertension. Some hormonal components are also capable of regulating the activity of VDCCs, and thus affect cellular functions. A more detailed discussion is presented in the later part of this chapter.

5.1.2. Calcium homeostasis and neurodegenerative cytotoxicity

$[Ca^{++}]_i$ regulates diverse neuronal processes, including development and maturation, gene expression, cell death, synaptic plasticity, transmitter release, excitability, etc. The pervasive involvement of Ca^{++} in neuronal function suggests that altered Ca^{++} homeostasis may be the fundamental mediator of age-related changes in the nervous system.

The dysregulation of cellular calcium homeostasis in aging has been observed on multiple cellular levels. Cellular disruption may involve abnormal functions in endogenous buffering mechanisms and Ca^{++} binding proteins, extrusion and sequestration pumps, membrane channels, intracellular storage sites, and intracellular release channels/receptors.

Decreases in $[Ca^{++}]_i$ transients with age have been reported in dentate granule cells [12, 382] and dorsal root ganglion neurons. [248] Elevated basal $[Ca^{++}]_i$ and reduced $[Ca^{++}]_i$ transients have been described in several types of neurons and synaptosomes from aged rat brain. [234, 352, 573] Increased $[Ca^{++}]_i$ has been observed in aged adrenergic neurons [86, 159] as well. Age-related increases in Ca^{++} influx have been demonstrated in hippocampal CA1 pyramidal neurons and in basal forebrain neurons, [234] suggesting impaired Ca^{++} homeostasis with age. Decreased Ca^{++} buffering with age has been proposed to explain these findings. In contrast, an age-related decrease in basal $[Ca^{++}]_i$ in various rat and mouse neurons has implied reduced Ca^{++} influx and increased buffering.

[263] Most observations from the available literature agree that some form of subtle Ca^{++} dysregulation is a critical factor in brain aging. In most, but not all, cases the specific form of the dysregulation has tended to suggest a condition of elevated basal $[Ca^{++}]_i$. The mechanisms of increased $[Ca^{++}]_i$ induced neuronal cytotoxicity have been discussed in Chapter1. However, the decrease in basal $[Ca^{++}]_i$ seen in some studies suggests a diminishing cellular function in aged neurons.

Evidence for loss of neuronal homeostasis and an excitotoxic contribution to neuronal degeneration in AD is considerable and rapidly increasing. [263, 267, 363, 369, 386, 455] The accumulation of Aβ has always been hypothesized to be causally involved in AD neuronal degeneration. It has been observed that Aβ is neurotoxic when it forms aggregates. It has also been revealed that the mechanisms of Aβ neurotoxicity apparently involve induction of free radical production in neurons, and disruption of $[Ca^{++}]_i$ – regulating mechanisms. This results in aberrant elevation of $[Ca^{++}]_i$ and increased sensitivity to excitatory stimuli. [285, 376, 380, 388] Exposure of cultured human cerebrocortical or rat hippocampal neurons to Aβ resulted in progressive elevation of $[Ca^{++}]_i$. [388] The time course of $[Ca^{++}]_i$ elevation is on the order of hours to days. That aggregated Aβ induced $[Ca^{++}]_i$ elevation is observed at the single cell level. Neurons with large amount of Aβ associated with their cell surface have a higher $[Ca^{++}]_i$ than neurons with little Aβ associated with their surface. Importantly, neurons exposed to Aβ (for only a few hours) exhibit enhanced $[Ca^{++}]_i$ responses to excitatory stimuli including glutamate and membrane depolarization with KCl. [362, 380] This increased sensitivity to excitation is correlated with greatly increased vulnerability to excitotoxicity. Interestingly, the $[Ca^{++}]_i$

-destabilizing action of Aβ may not be limited to neurons. Elevation of $[Ca^{++}]_i$ has been observed with lymphocytes and in oocytes exposed to Aβ. [371]

The site of action of Aβ in disrupting calcium homeostasis appears to be the plasma membrane since the aggregates of Aβ accumulate at the membrane and Aβ toxicity is experimentally attenuated when Ca^{++} influx is reduced. [388] Previous experimental data suggest that Aβ is capable of forming cation-selective pores in lipid bilayers. [16, 312, 541] These peptide channels have similar ionic conductance to HVA channels of VDCCs, and preferentially pass Ca^{++}. Additional studies indicate that Aβ induces Ca^{++} influx via existing channels, including L-type channels. [124, 164] Increased Ca^{++} influxes, with resultant disruption of calcium homeostasis, may be the underlying cause of Aβ neurotoxicity and ultimate neurodegeneration.

In PD, the precise cause and mechanisms involved in the selective dopaminergic neuronal loss are not fully understood. It has been suggested that apoptosis may be the end result in a series of events leading to neuronal death in PD. [373, 506, 538] There have been few attempts to inhibit apoptosis in PD. Deprenyl has been shown to enhance the transcription of *Bcl-2*, which protected cells from apoptosis. [550] Another component of the apoptotic cascade that is potentially amenable to therapeutic intervention is the transmembrane flux of Ca^{++}. Direct links between PD etiology and dysfunction of calcium homeostasis are lacking. However, there are several lines of evidence at the cellular and molecular levels that support the notion that calcium antagonists may potentially affect PD favorably. Studies with animals have shown that the calcium channel density on neurons dramatically increases with aging. [553] Extrapolating to humans, PD patients, most of whom are elderly, may have increased vulnerability to calcium related neurotoxicity, due to an increased channel density. Calcium channel antagonists have been shown to block the development of experimental Parkinsonism in a few studies. Nimodipine, the L-type VDCC blocker, blocks MPTP induced Parkinsonism in mice [332] and non-human primates. [333] However, the effects of nimodipine observed in the above two studies were at the SN, but not at the striatal level, implicating differential mechanisms of action of MPTP-induced neurotoxicity at the nigral versus the striatal level. It is not clear if the precise mechanisms of the observed phenomenon of nimodipine are through calcium channel blockade. Some research proposals focus largely on the neuroprotective potential of calcium channel blockers as inhibitors of apoptosis in PD. There is, thus far, no definitive evidence suggesting a neuroprotective effect of calcium antagonists, and no calcium channel blockers have been used in PD therapeutics. Further study of how stabilizing cellular calcium homeostasis can affect apoptotic cell death in neurodegeneration may lead to new ideas in PD treatment.

5.1.3. Estrogen and calcium homeostasis

Estrogen has long been considered a key factor in controlling calcium metabolism. Estrogen, together with 1,25-dihydroxyvitamin D, and parathyroid hormone, influence bone metabolism. In normal women, estrogen withdrawal increases bone resorption and causes a rise in serum calcium. Estrogen deficiency after menopause contributes greatly to the high incidence of osteoporosis in postmenopausal women. [18, 28, 57] The cardiovascular system is also influenced by the steroid hormones. Women are less susceptible to cardiovascular disease before the onset of menopause, when cessation of ovarian hormone production is accompanied by an increased incidence of coronary heart diseases. [554, 634] The beneficial effects of hormone replacement therapy are now well recognized. Activation of genomic ERs results in inhibition of vascular smooth muscle cell proliferation and up-regulation of constitutive NOS in endothelial cells. [202, 398] In addition to the genomic actions of steroid hormones in the vasculature, recent evidence confirms that E2 acutely restores impaired coronary blood flow in postmenopausal women. The consensus of several studies is that acute vascular relaxation induced by E2 is predominantly endothelium-independent and mediated by the inhibition of Ca^{++} influx through L-type Ca^{++} channels. This results in decreases in myosin light chain kinase phosphorylation and contraction of smooth muscle. [323, 428, 627] Moreover, in addition to inhibiting voltage-operated Ca^{++} channels in smooth muscle cells, E2 has also been reported to increase Ca^{++}-activated K^{+} currents. [594] Thus, activation of genomic and nongenomic steroid receptors by E2 modulates vascular function through the modulation of cellular ion homeostasis.

Most studies have reported that the effects of estrogen in regulating neuronal calcium homeostasis are non-genomic. Mermelstein *et al.* [399] reported that physiological concentrations of E2 decreased Ba^{++} currents in neostriatal neurons through L-type channels via activation of a G-protein signaling pathway. The modulation was sex specific since the effect was greater in female neostriatal neurons than in cells taken from male rats. L-type channels are found primarily in somatic and proximal dendritic membranes. [268, 591] By decreasing L-type currents, E2 may affect spike generation, patterning of the neurons, and also gene transcription [20]. The authors suggested that G-protein activation may induce long-term intracellular changes via phosphorylation. Therefore, the non-genomic action of E2 may also have a long term effects on neostriatal function. Kim *et al.* [319] observed that in PC12 cells, E2 itself did not show any effect on

$[Ca^{++}]_i$, whereas 70mM K^+ did not induce a prominent rise in $[Ca^{++}]_i$, in the presence of E2. They found that the actions of E2 in PC12 cells were through the blockade of both N- and L-type VDCCs. Although L- and N-type channels appeared to be blocked to about the same extent, L-type VDCCs seem to be the main target of the E2-induced inhibition because L-type VDCCs compose the major portion of the E2-sensitive Ca^{++} influx through VDCCs.

Although E2 has been shown to block Ca^{++} entry through VDCCs, other results indicated that in E2 treated ovariectomized rats, the amplitude of Ca^{++} currents was increased in CA1 hippocampal neurons. The enhanced Ca^{++} influx may take place in the newly developing dendritic spines, due to the steroid-induced expression of Ca^{++} channels. [306]

The available literature has shown that estrogen is capable of modulating a large array of biological responses through the regulation of calcium homeostasis involving VDCCs. In the nervous system, Ca^{++} acts as an important second messenger in regulating neurotransmitter release, synaptic activity, and even neuronal survival. The regulation of calcium homeostasis may be an important aspect of the neuroprotective actions of estrogen, in neurodegenerative disorders.

5.2. Experimental design

The experiments were designed to investigate whether the protective effects of estrogen in the observed cytotoxic models occurred through stabilizing cellular calcium homeostasis. In most of this part of the study, we focused only on the Aβ and MPTP models, the insults closely associated with AD and PD.

5.2.1. The effects of calcium channel antagonists in Aβ- and MPTP-treated SK-N-SH cells

Several VDCC antagonists were studied in Aβ and MPTP models. Upon initiation of experiments, the L-type blocker, nifedipine (5μM), the N-type channel antagonist, ω-conotoxin (1μM), or the non-selective T-type channel blockers $NiCl_2$ (1μM) and flunarizine (0.1μM), were added to SK-N-SH cell culture with Aβ (20μM) or MPTP (0.75mM). Cell viability was assayed after 96hr. The concentration chosen for each blocker was based on the effective concentrations that block Ca^{++} entry through the relevant VDCCs in patch clamp studies in the literature. E2 was tested simultaneously for comparison.

5.2.2. The effect of extracellular [Ca^{++}] on Aβ- and MPTP-treated cells

To determine the effects of decreased extracellular [Ca^{++}] on the Aβ and MPTP models, a series of calcium concentrations were modified in the culture media. The concentrations of the $CaCl_2$ in MEM in the current study were 0, 0.1, 0.5, and 1mM. The $CaCl_2$ concentration in culture media is usually 1mM. By decreasing the concentration of $CaCl_2$ in culture media, the source of Ca^{++} influx was diminished. Cell viability was assayed after 96hr.

5.2.3. Ca^{++} channel activity

The effects of Aβ (20μM) or MPTP (0.75mM) on VDCC activity were tested with SK-N-SH cells using the whole cell version of the patch clamp technique. SK-N-SH cells were cultured with Aβ or MPTP for 96hr. Cells were then tested for channel activity. GH3 cells (rat pituitary tumor cells) were used as a positive control to compare the channel activities.

5.2.4. $[Ca^{++}]_i$ determination by the fura-2 method

To study the effects of Aβ and MPTP on the $[Ca^{++}]_i$ increment induced by KCl (30mM), SK-N-SH cells were cultured with Aβ (20μM) or MPTP (0.75mM) for 96hr. The cells were challenged by 30mM KCl, and the $[Ca^{++}]_i$ increment was recorded. GH3 cells, used as controls, were tested under the same conditions.

5.2.5. Investigation of L-type Ca^{++} channel expression by Western blot analysis

To investigate how Aβ and MPTP modify L-type Ca^{++} channel expression, Western blotting with antibodies against α_1 subunits was performed. SK-N-SH cells were cultured with Aβ (20μM) or MPTP (0.75mM) for 96hr. Neuroprotective agents as revealed by cell viability studies were added to Aβ- and MPTP-treated cultures. Cells were harvested after 96hr, and protein samples were prepared from the cells. We aimed to investigate the functional L-channel expression in different treatment groups, and also determine which isoform of the neuronal L-channel was modified by each insult. The antibodies used in the current study were anti-DHP binding complex (α_1-subunit) monoclonal antibody (anti-α_1) (BioMol Research Laboratories, Inc., Plymouth Meeting, PA, USA), which recognizes functional L channels, and anti- neuronal L-type Ca^{++} channel α_{1C} subunit and α_{1D} subunit (anti-α_{1C} and anti-α_{1D}) polyclonal antibodies. The anti-α_1 is a monoclonal antibody that immunoprecipitates the DHP binding complex which recognizes a novel protein of 170-220KDa. It is reactive with L-type Ca^{++} channel α_1 subunit, and the addition of anti-α_1 blocked the DHP sensitive VDCCs in BC3H1 cells.[416] Anti-α_1 should recognize any isoforms of the α_1 subunits non-specifically. Anti-α_{1C} wais developed using a synthetic peptide corresponding to amino acids 818-835 of the α_{1C} subunit of rat brain voltage-gated calcium channel (VDCC, CNC1) with additional N-terminal lysine and tyrosine, conjugated to KLH, as immunogen, and the antibody recognizes α_{1C} by immunoblotting. Anti-α_{1D} is raised against a synthetic peptide corresponding to amino acids 809-825 of the α_{1D} subunit of rat brain VDCC (CND1), with additional N-terminal lysine and tyrosine conjugated to KLH. The antibody recognizes the α_{1D} subunit of L-type VDCCs by immunoblotting and immunocytochemistry.

Densitometry analysis was performed to determine the changes in channel protein expression.

5.3. Results

5.3.1. The effects of Ca^{++} channel blockers on Aβ- and MPTP-treated SK-N-SH cells

As observed previously, Aβ (20μM) induced cell death after 96hr culture. In the presence of the L-type calcium channel blocker, nifedipine (5μM), the cell viability loss caused by Aβ was almost fully prevented. The other VDCC antagonists, including the N-type channel antagonist, ω-conotoxin (1μM), and the non-selective T-type channel blockers, $NiCl_2$ (1μM) and flunarizine (0.1μM) did not change the Aβ induced cytotoxicity (Fig. 5.1). These data suggested that Ca^{++} influxes through L-type VDCCs, but not through other channels, might contribute to Aβ induced neuronal injury in SK-N-

SH cells, and that nifedipine, by attenuating such influxes, was able to attenuate Aβ toxicity.

In MPTP-treated SK-N-SH cell culture (Fig. 5.2), none of the channel blockers showed any protective effect against MPTP induced cell death, suggesting that the cytotoxicity in this model probably not involved modulation of Ca^{++} influxes through VDCCs.

A dysregulation of cellular calcium homeostasis could contribute to neuronal cell death through different mechanisms, involving both apoptosis and necrosis. Nifedipine, an L-type VDCC blocker, provided significant protection against neuronal death induced by Aβ in SK-N-SH cell culture. This suggests that L-type VDCCs played an important role in maintaining neuronal calcium homeostasis for survival and function, and correlated with both apoptosis and necrosis in this cell line. Further studies to determine the correlation among calcium homeostasis, neuronal toxicity, and the neuroprotective effects of estrogen focused on Aβ and MPTP models only, since these two models are closely associated with specific neurodegenerative disorders, and since E2 was able to protect in both models.

5.3.2. Extracellular [Ca^{++}] and neuroprotection

To further determine that the source of the altered calcium homeostasis in the cytotoxic models is extracellular Ca^{++}, a series of calcium concentrations were used in the culture media. The concentrations of the $CaCl_2$ in MEM in this study were 0, 0.1, 0.5, and 1mM. The $CaCl_2$ concentration in culture media is usually 1mM. By decreasing the concentration of $CaCl_2$ in the culture media, the source of Ca^{++} influx is diminished. In Aβ-treated SK-N-SH cells, a decrease of extracellular calcium by 90% significantly protected against neuronal death (Fig. 5.3). However, the degree of protection caused by reducing extracellular $CaCl_2$ was not as profound as that of that of nifedipine. The calcium-free media in the Aβ model did not show any protective effects, suggesting that the proper amount of extracellular Ca^{++} was essential for SK-N-SH cells. In the MPTP treated cultures, the modification of $CaCl_2$ in the media did not change the cell viability loss. These data suggested that the toxic effects of Aβ in SK-N-SH cells involved dysregulation of cellular calcium homeostasis, and the dysregulation was at least partially due to an excess influx of Ca^{++} from the extracellular calcium pool. Previous reports on Aβ and βAPP toxicity also indicated that removal of extracellular calcium was beneficial to neurons.[374, 541]

5.3.3. Ca^{++} channel acitivity and $[Ca^{++}]_i$ determination

To investigate how the cytotoxic treatments affected Ca^{++} channel activity, patch clamp with whole-cell recording was performed. In both control culture and 96hr Aβ (20μM)-treated SK-N-SH cells, no tracing of either L-type or N-type currents could be recorded (Fig. 5.4, A~C). The test with fura-2 methods to measure intracellular calcium responses to depolarization yielded similar results. SK-N-SH cells did not respond to 30mM KCl stimulated depolarization (Fig. 5.5, A~C). To rule out the possibility that the methods used in the current studies might be the problem, a comparison study with GH3 cells was carried out with the same equipment and settings. L-type Ca^{++} channel activities were clearly recorded, and the channels were identified by their amplifying response to Bay K8644 and inhibitory responses to nifedipine (Fig. 5.6, A~C). In addition, GH3 cells demonstrated a dramatic rise in $[Ca^{++}]_i$ in response to KCl stimulation with the fura-2 method, and this was recorded (Fig. 5.7, A~C).

5.3.4. L-type Ca^{++} channel expression by Western blot analysis

We have found that the presence of the L-type VDCC blocker, nifedipine, and diminished extracellular Ca^{++} were protective in Aβ induced neuronal toxicity in SK-N-SH cells. The function and the expression of the L-type channel may play a key role in regulating the balance between neuronal survival and death. Moreover, E2 acted as a neuroprotectant in these cytotoxic models. The protective effects of E2 and the VDCC blocker could be related to the expression of the channel, although it has not been previously demonstrated in the literature.

Western blot analysis showed that Aβ caused an overexpression of the L-type channel proteins. Both E2 (0.1nM) and nifedipine (5μM) were able to inhibit the overexpression of α_1, α_{1C} and α_{1D} in Aβ treated SK-N-SH cell culture (Fig. 5.8 ~ 10). However, the increased α_{1C} and a_{1D} expression was not modified by either E2 or nifedipine in MPTP models (Fig. 5.9 ~10). Furthermore, MPTP caused an underexpression of α_1-DHP binding complex protein. Estrogen or nifedipine alone had no effect on normal untreated cells.

To determine which L-type VDCC subunits were regulated by the cytotoxic insults, we first demonstrated the expression of the α_1 subunit, and other two neuronal isoforms, α_{1C} and α_{1D}, in SK-N-SH cells. Anti-α_1 recognized a 215KDa protein in SK-N-SH cells. However, the expression of the protein was low in the basal condition. Anti-α_{1C} reacted with a 165Kda protein, and anti-α_{1D} recognized a 175KDa protein, respectively. As detected by Western blot analysis, α_1 (DHP binding complex) expression in SK-N-SH cells increased by 36.1 ± 3.44 % (Fig. 5.8 A and B) with an exposure to Aβ (20μM). Both E2 and nifedipine significantly reversed the overexpression. In contrast, MPTP treatment decreased the production of α_1 protein by 37.51 ± 7.26 % (Fig. 5.8, A &B), and neither E2 nor nifedipine were capable of modifying the change. Aβ increased protein expression by 72.4 ± 4.45 % as recognized by anti-α_{1C} in the culture, and both E2 and nifedipine significantly diminished the increment (Fig. 5.9). In MPTP-treated groups, an increment in α_{1C} was observed as well. However, the addition of E2 or nifedipine did not change the magnitude of the increment (Fig. 5.9). An overexpression of α_{1D} was also detected using anti-α_{1D} antibody in Aβ treated SK-N-SH cells. As seen in α_1 and α_{1C}, both E2 and nifedipine significantly restored the overexpression of α_{1D}. In addition, MPTP treatment dramatically amplified the expression of α_{1D}. However, neither E2 nor nifedipine had any effect on MPTP-treated culture (Fig. 5.10). These observations highly correlated with data from cell viability studies with Ca^{++} channel blockers showing that nifedipine protected SK-N-SH cells in Aβ induced cytotoxicity, but not in MPTP-treated cultures.

5.4. Disscussion

Estrogen has been shown to exert neuroprotective effects in various studies involving various mechanisms. We have observed that E2 was capable of protecting SK-N-SH cells from neurodegenerative insults such as Aβ and MPTP, and high density culture and serum deprivation through anti-apoptotic and anti-necrotic pathways. Cellular calcium homeostasis disruption is a hallmark of both apoptosis and necrosis. The ability of estrogen to regulate biological responses at multiple cellular levels, including stabilizing calcium disregulation, implies the possibility that estrogen can induce neuroprotection through regulation of cellular calcium. To investigate how calcium regulation was affected in the 4 cytotoxic models, we tested the effects of VDCC blockers on cell viability, L-type channel functions, and expressions of channel proteins.

The cell viability study with VDCC blockers has shown that the L-type calcium channel blocker, nifedipine (5μM), induced profound protective effects on cell viability loss caused by Aβ. In this model, nifedipine almost fully restored the loss of live cells. However, the other VDCC antagonists, including the N-type channel antagonist, ω-conotoxin (1μM), the non-selective T-type channel blockers, $NiCl_2$ (1μM) and flunarizine (0.1μM) did not change the cell viability. These data suggested that L-type channels predominated in SK-N-SH cells, and that the cytotoxic effects of Aβ might at least partially involve the dysregulation of calcium homeostasis via excessive influxes through L-type VDCCs. The presence of DHPs (including nifedipine) might limit calcium entry, and exert protective effects against Aβ induced cytotoxicity.

From the available literature and our observations, one could postulate that the Aβ cytotoxicity was due to calcium homeostasis disruption through excessive Ca^{++} influxes via L-type VDCCs. Controlling Ca^{++} influxes in the Aβ model could reverse the cytotoxic effects as revealed by the nifedipine effects and diminishing the extracellular calcium pool. To investigate the how L channel activity changes in SK-N-SH cells after exposure to Aβ and MPTP, the patch clamp technique and fura-2 method were used in the current studies. Unfortunately, neither technique was suitable for recording the channel activity changes in cell death models with SK-N-SH cells. In the current available literature, only Lo and colleagues [346, 347] have presented calcium channel data with patch clamp techniques in SK-N-SH cells. However, they have indicated that SK-N-SH cells expressed only low basal levels of voltage-gated ionic currents. Moreover, retinoic acid (RA) was supplied to the culture to induce differentiation and cellular responsiveness to neurotrophins. However, the addition of RA might change the metabolism and the functions of the cells. It has been shown that RA may act as a type of signal molecule working through a nuclear receptor in the regulation of regional differentiation of the central nervous system [54] through the modification of expression of neuronal cell surface receptors to peptide growth factors. An *in vitro* induction of differentiation of neuroblastoma cells by RA was linked to a rapid decrease of phosphatidylinositol turnover. [463] RA can induce both differentiation and apoptosis. The induced apoptosis was through caspase dependent pathways in human neuroblastoma cell lines and in esophageal cancer cell lines. [463, 585, 614] Moreover, RA increased mitochondrial membrane permeability and stimulated the release of cytochrome c. [252] The current study investigated genomic responses with both apoptotic and necrotic cell death models; the additional features of RA might largely interfere with the neuronal toxic and neuroprotective properties. The fact that Ca^{++} channels and KCl-induced Ca^{++} influx could not be recorded was probably due to the following reasons: (1) Voltage-gated currents were expressed at very low levels in the basal condition, and without the induction of neuronal differentiation, they were hard to record. (2) Cell death insults (induced apoptosis or necrosis) may induce an overexpression of the channels to promote Ca^{++} influx. However, with the patch clamp technique, only healthy cells, with intact membranes were patched on. Condensed cells or cells with blebbing membranes could not be recorded. With the fura-2 method, the fluorimeter system was equipped with a flow through perfusion system which constantly perfused and removed the recording buffer in the chamber. The dead cells or the dying cells might have detached from the adhesive surface and largely been removed by the washing and the flow through procedures (cells are washed three times with extracellular solution before patching on in patch clamp studies, and they were under constant perfusion with the flow through procedures in fura-2 studies). The responses which were recorded were from intact cells. Therefore, the patch clamp and fura-2 methods were not suitable for investigating the cellular mechanisms in current cytotoxic models. (3) Positive recordings on GH3 cells in both systems suggested the methods were sound and the problems might reside in the model as suggested above.

How Aβ affects calcium homeostasis has been discussed in detail in chapter 1 and chapter 3. In general, the mechanism of Aβ neurotoxicity apparently involves induction of free radical production in neurons and disruption of $[Ca^{++}]_i$ regulating mechanisms, resulting in aberrant elevations of $[Ca^{++}]_i$ and increased sensitivity to excitatory stimuli. Exposure of cultured human cerebrocortical [221, 431] or rat hippocampal neurons [3, 13, 40] to Aβ resulted in progressive elevation of $[Ca^{++}]_i$. Neurons exposed to Aβ exhibited enhanced $[Ca^{++}]_i$ responses to excitatory stimuli including glutamate [10, 79, 245] and membrane depolarization with KCl. [466, 559] This increased sensitivity to excitation was correlated with greatly increased vulnerability to Aβ. Aβ also rendered neurons more vulnerable to hypoglycemic injury. These data supported a role for Aβ in an excitotoxic mechanism of neuronal degeneration in AD. It seems likely that disruption of $[Ca^{++}]_i$ homeostasis by aggregated Aβ results from nonspecific effects at the plasma membrane.

For example, Aβ might perturb the regulation of Ca^{++} channels or glutamate receptors resulting in increased Ca^{++} influx, or Aβ might impair Ca^{++} removal from the cell.

The observation that nifedipine could induce neuroprotective effects against Aβ correlated very well with previous reports. Protective actions by L-type VDCC antagonists in Aβ induced neuronal toxicity in rat cortical neurons [590] and cholinergic neurons in the magnocellular nucleus basalis [256] have been reported previously. Weiss *et al.* [590] demonstrated that in primary cultures of murine cortical neurons, exposure of cultures to Aβ (3 ~ 25μM) generally triggered a slow, concentration-dependent neurodegeneration (over 24-72 h). With submaximal Aβ exposure (10μM), substantial (> 40% within 48 h) degeneration often occurred and was markedly attenuated by the presence of the Ca^{++} channel blocker nimodipine (1 ~ 20μM) during exposure to Aβ. Sberna *et al.* [508] observed that Aβ (25 -35) increased AChE activity in P19 embryonic carcinoma cells. To examine the possibility that the increase in AChE expression was mediated by an influx of calcium through VDCCs, drugs acting on VDCCs were tested for their effects. Inhibitors of L- type VDCCs (diltiazem, nifedipine, and verapamil), but not blockers of N- or P- or Q-type VDCCs, resulted in a decrease in AChE expression. Agonists of L- type VDCCs (maitotoxin and S(-)-Bay K 8644) increased AChE expression. In addition, Aβ has been reported to potentiate a nimodipine-sensitive L-type barium conductance in N1E-115, a murine neuroblastoma cell line as detected by patch clamp technique. This suggested that the neurotoxic effects of Aβ might be mediated by its ability to form cation channels *de novo*, or alter the activity of cation channels already present in the cell membrane. These observations, together with ours indicated that Ca^{++}

influx through voltage-gated channels may contribute to Aβ-induced neuronal injury and that DHPs, by attenuating such influx, were able to attenuate Aβ neurotoxicity.

None of the calcium channel blockers exerted any effects on either the MPTP model, suggesting that the dysregulation of calcium metabolism was not the dominant role in cell death in this model. However, a change in $[Ca^{++}]_i$ could be the downstream phenomenon when cell death occurred.

The site of action of Aβ in disrupting neuronal $[Ca^{++}]_i$ homeostasis appeared to be the plasma membrane because Aβ toxicity was attenuated when Ca^{++} influx through plasma membrane was experimentally reduced. When extracellular calcium concentration was diminished by decreasing $CaCl_2$ concentration (0.1mM, instead of 1mM) in the cell culture media, cell viability was significantly restored in Aβ-treated SK-N-SH cells, but not in MPTP-treated culture. These data confirmed what was observed with the Ca^{++} channel blockers, suggesting that Aβ-, but not MPTP-induced cytotoxicity was through calcium homeostasis disruption. Suh *et.al.* [541] reported a recombinant carboxy-terminal 105 amino acid fragment (CT 105) of APP, the precursor protein of Aβ, did not change $[Ca^{++}]_i$ in calcium-free media, whereas cells in normal media treated with 10μM CT 105 for 6hr showed a significant increase in calcium concentration, suggesting that calcium may enter the cells from the outside. Also, cells in the calcium-deficient medium were protected against the neurotoxicity of CT 105. Thus, calcium influx should be necessary for the neuronal damage induced by CT 105. Mattson [370] in an earlier report indicated that calcium influxes were mechanically involved in Aβ cytotoxicity since removal of extracellular calcium could protect neurons against Aβ toxicity.

Based on the observations that nifedipine restored cell viability in the Aβ model, and decreased extracellular Ca^{++} concentration protected Aβ induced cell death, a question was raised whether functional changes of the existing channels could account for the toxicity of Aβ. To address that question, the expression of L-type VDCCs was investigated by analyzing the protein expression of the α_1 subunit. Moreover, whether the protective effects of E2 and nifedipine on Aβ- and MPTP-induced cytotoxicity acted through mechanisms related to L channel expression was also investigated. Results have shown that with the anti-α_1 DHP binding complex antibody, low expression was detected under basal conditions in SK-N-SH cells. An increased expression of the channel protein was found in Aβ treated culture, and both E2 and nifedipine were able to inhibit the increased expression, while neither E2 nor nifedipine modify the expression in normal cells. It has been suggested that aggregated Aβ appeared to act as Ca^{++} channels in cell membranes. [16, 312] Aβ (1~ 40) exhibited ionophore-like properties in human erythrocytes and pore structures were formed in synthetic bilayers. [16, 367, 468] Moreover, the increase of intracellular Ca^{++} after amyloid infusion was found to be slow and required aggregation of amyloid. Therefore, it seems unlikely that a direct activation of receptors or channels was involved. [374] Our results demonstrated that the Aβ treatment could increase expression of L-type VDCCs, and the elevated Ca^{++} influxes could probably be through the new synthesized and preexisting L channels. The new channel protein synthesis may be a confirmation of the previous observed pore formation property of Aβ at plasma membrane. This phenomenon of the Aβ might also account for an important aspect of the cytotoxicity, probably due to a change in the pattern of Ca^{++} entry. These results contributed to a further understanding of the pathological cascades of Aβ toxicity at the subcellular level, and might be helpful in developing new therapeutic strategies in AD treatment.

E2 has been shown to be neuroprotective in a wide variety of studies. The ability of E2 to regulate calcium homeostasis has been previously reported [217]. In Aβ toxicity studies, Goodman *et al.* [217] observed that estrogens attenuated Aβ- and glutamate-induced elevation of intracellular free Ca^{++} concentrations. They concluded that estrogens could directly affect neuronal vulnerability to excitotoxic, metabolic, and oxidative insults, suggesting roles for the steroid in neurodegenerative disorders. In addition, E2 has been reported to abolish the positive modulatory effect of the L-type VGCC agonist, Bay K8644, in rat hippocampal neurons, suggesting an action primarily at L-type VDCCs; [334] it also inhibited the NMDA-induced $[Ca^{++}]_i$ increases. It has been reported that E2 plays a protective role in brain ischemic injuries by regulating the vasculature tone. High doses of E2 protected neurons from ischemia by inhibiting the release of Ca^{++} from the intracellular Ca^{++} stores, as well as by inhibiting the influx of Ca^{++} from the extracellular space.hippocampal CA1 pyramidal cells in gerbil. [103] However, most studies revealed the non-genomic effects of E2 in antagonizing VDCCs. In the current studies, E2 and nifedipine have been found to restore the Aβ induced cytotoxicity. The magnitude of the protection by E2 in the Aβ model was similar to that of nifedipine in cell viability studies. Moreover, as detected by Western blot analysis with anti-α_1, E2 also significantly reversed the increase of α_1 expression, in a manner similar to that of nifedipine. In the Aβ model, the protective effects exerted by E2 and nifedipine could, in fact, be through stabilizing calcium homeostasis via maintaining the physiological level of the L-type channels expressed in the neuronal plasma membrane of SK-N-SH cells.

How E2 affects neurodegenerative changes via Ca^{++} mediated pathways has not been clearly illustrated. The current observation that E2 protected Aβ induced neuronal cell death through a reversal of excessive L-type VDCC expression is novel.

The observed protective effects of DHP in Aβ models could be partially due to its ability to block the Ca^{++} entry. However, the observation that nifedipine protected neurons against Aβ cytotoxicity through restoration of the overexpression of L-type VDCCs suggested that the protective action was through a genomic effect of this channel blocker. It can be hypothesized that the predominant protection exerted by nifedipine in the Aβ model was mediated through down-regulation of L-type channel protein expression rather than its novel acute blockade of Ca^{++} entry. This is the first demonstration that DHPs are capable of regulating L-type VDCC protein expression when the protein expression is elevated under certain pathological conditions. It can be proposed that when cell death occurs, the membrane permeability may have changed to allow the access of nifedipine. By binding to certain cytosolic or nuclear components, nifedipine may be able to modulate gene regulation and protein expression. This genomic action of nifedipine can help to better explain the previously observed neuroprotective effects of DHPs in the Aβ models and other brain injuries. A new fundamental role of DHPs will be considered in regulations of calcium homeostasis.

In many neurodegenerative studies, apoptosis, a programmed cell death, has been observed to depend on $[Ca^{++}]_i$ changes. In brains of patients with AD, the apoptosis-inducing proteins, such as p53 [322] were increased. Aβ (25 ~ 35) induced apoptosis in a Ca^{++}-dependent manner in several *in vitro* studies. [69, 82, 123] Part of the Aβ-induced Ca^{++}-dependent neurotoxicity may be due to triggering the apoptosis program in neurons.

However, in our study, Aβ induced necrosis in SK-N-SH cells. The observed actions of E2 and nifedipine indicated that calcium homeostasis disruption was involved in neuronal necrotic processes as well. The protection against Aβ induced necrosis exerted by E2 and nifedipine was an anti-necrotic effect through stabilizing cellular calcium homeostasis. Experiments investigating two isotypes of the α_1 subunit, α_{1C} and α_{1D} with relevant antibodies have shown results similar to those seen with anti-α_1 DHP binding complex, suggesting that α_{1C} and α_{1D} were both overexpressed with Aβ treatment in SK-N-SH cells. E2 and nifedipine could regulate the overexpression of both isotypes.

In MPTP treated groups, α_1 expression was decreased. In contrast, the expressions of α_{1C} and α_{1D} were elevated with the presence of MPTP. Neither E2 nor nifedipine showed any effects on channel protein expression in MPTP treatment groups. The failure of nifedipine to regulate α_1, α_{1C}, and α_{1D} subunit expressions correlated with our results in the cell viability study. E2 did not modify the changes in α_{1C} and α_{1D} expression, but protected neurons from MPTP induced toxicity, probably through other anti-apoptotic pathways. E2 might have regulated calcium dysfunction associated with apoptosis in MPTP induced cell death through other up- or downstream cascades rather than regulating the L-type channel expression. However, in one study by Fass *et al.*,[175] the molecular basis of long-lasting suppression of Ca^{++} channel current in pituitary melanotropes by chronic dopamine exposure was examined. Experiments involving *in vivo* and *in vitro* treatments with the dopaminergic drugs haloperidol, bromocriptine, and quinpirole showed that D2 receptors stimulation persistently decreased α_{1D} L-type Ca^{++} channel mRNA and L-type Ca^{++} channel currents without altering channel gating properties. Our findings that α_{1D} expression was up-regulated by MPTP, a dopaminergic toxin, somewhat correlated with Fass's observation that dopaminergic agonists down-regulated α_{1D}.

Observations on channel protein expression in the MPTP model might seem conflicting. A decrease of α_1 DHP binding complex and increases in, α_{1C} and α_{1D}, the two isotypes in MPTP treatment groups have been found. It can be proposed that the α_1 DHP binding complex acted as a functional L channel in this cell line. A decrease in protein expression might suggest a diminished cellular function in the presence of adopaminergic toxin. The elevated α_{1C} and α_{1D} expression that could not be regulated by either E2 or nifedipine was probably a non-specific response related to MPTP effects. α_{1C} and α_{1D} might also be non-functional channels in MPTP treated cells, or channels insensitive to nifedipine or E2. The increment could be hypothesized to be related to a generalized increase in protein synthesis associated with apoptotic cell death. However, it could not be related to increased Ca^{++} entry, because Ca^{++} removal from media did not protect the cells. However, these channels might be related to other parts of cellular calcium regulation, such as membrane channels of organelles of intracellular stores.

Although significant advances have been made in the symptomatic treatment of PD, effective neuroprotective therapies have not yet been perfected. The role of calcium in apoptotic neurotoxicity is clear. Calcium antagonists may then have a protective effect in PD. Several lines of evidence support this notion, including the reported effect of calcium antagonists on other human diseases involving apoptosis. Animal studies demonstrated the ability of calcium antagonists to protect SN neurons from the toxin MPTP. The common calcium antagonists in use for the treatment of cardiovascular diseases have the potential to modify calcium neurotoxicity in PD since they block the L-type calcium channels predominantly responsible for regulating intracellular calcium in midbrain dopaminergic cells. However, there is at present no clear evidence suggesting a neuroprotective effect of calcium channel blockers in PD. [484] Our present data with nifedipine indicated that DHPs may not be useful in PD. On the other hand, estrogen has shown a promising protective effect in dopaminergic degenerative disorders. The protective actions were largely related to ER-mediated anti-apoptotic effects and non-ER-mediated anti-oxidative processes. [505, 507] In MPTP induced SK-N-SH cell death, the protective effect of E2 acted through ERβ pathways.

In summary, Aβ induced necrosis in SK-N-SH cells was largely related to the dysregulation of calcium homeostasis. This is supported by the observations that incubation with L-type VDCC blocker, and decreased extracellular [Ca^{++}], could restore the cell viability loss induced by Aβ. Furthermore, the channel protein expression study with Western blot analysis has revealed that an increment in L-type channel α_1 subunit was associated with Aβ cytotoxicity. An increase in L-type VDCC expression induced by Aβ could mediate excessive Ca^{++} influx. This could be the major reason for Aβ pathology. E2 was able to reverse the overexpression of L channel proteins, and subsequently presumably controlled the Ca^{++} influx, and rescued the cell death. This process of E2 was through non-ER mediated pathways. Interestingly, administration of the acute L-channel blocker nifedipine showed a profound protective effect against Aβ by restoration of the L-channel overexpression as well. The blockade of Ca^{++} entry probably played a minor role in the protective process. These data are of great physiological significance in several aspects. First of all, the demonstration of the increment in L-type VDCC α_1 subunit overexpression induced by Aβ helps us to understand the cellular mechanisms related to Aβ cytotoxicity. A clear understanding of the toxicity cascade is essential for developing therapeutic compounds. Secondly, the protective effects of E2 in the Aβ model has been shown to involve stabilizing cellular calcium homeostasis, particularly through the maintenance of a normal level of L-type VDCC protein expression. This is a new feature of estrogen in neuroendocrine research concerning the genomic action of the female steroid hormone in the absence of ER activation. More interestingly, the L-type VDCC blocker, nifedipine, has shown promising protection in the Aβ model, very likely due to its capability restoring the overexpression of the L-type VDCC proteins in the presence of Aβ. Thus, nifedipine might prevent the excessive Ca^{++} influx induced cytotoxicity of Aβ by maintaining a physiological level of protein expression of L-type VDCC. This action is hypothesized to be mediated through its binding to certain cytosolic or nuclear components, and its exertion of protective effects through gene regulation and protein expression. This observation will help to explore new mechanisms and potential for DHPs in calcium homeostasis regulation, and in therapeutic aspects related to neuronal disorders and cardiovascular diseases.

E2, but not nifedipine, or diminished extracellular $[Ca^{++}]$, protected against MPTP induced apoptotic neuronal death in SK-N-SH cells through an ERβ mediated pathway. The protection against MPTP by E2 was probably due to anti-apoptotic and perhaps anti-oxidative properties of the steroid. In addition, MPTP treatment decreased expression of the α_1 DHP binding complex, but increased the expression of α_{1C} and α_{1D}; neither E2 nor nifedipine were able to modify the changes induced by MPTP. As detected in the previous study, Aβ induced cell death in SK-N-SH cells was through necrotic pathways, while the MPTP effects were through apoptosis. Further investigations will be needed to correlate the expression of different subunits with necrosis and apoptosis.

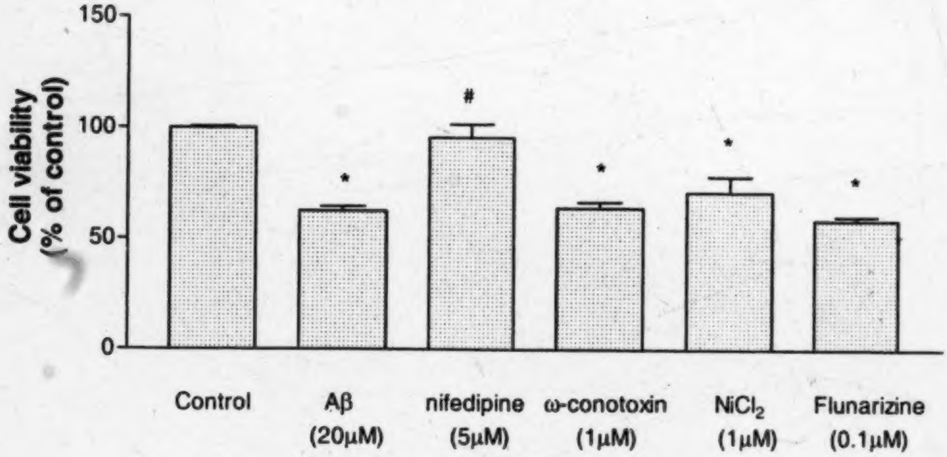


Fig. 5.1. The effects of Ca^{++} channel antagonists on Aβ (20μM)-treated SK-N-SH cells.

The channel blockers were added to each vial in the presence of Aβ when initiating experiments. Cell viability was assayed after 96hr culture.

*, $p<0.05$, compared with the control group;
#, $p<0.05$, compared to the Aβ treated group. (n=6)

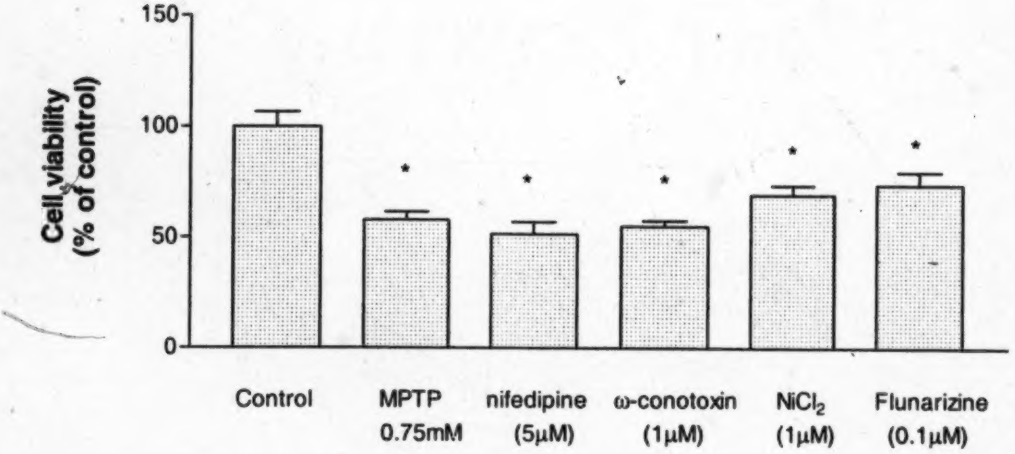


Fig. 5.2. The effects of Ca^{++} channel antagonists on MPTP (0.75mM)-treated SK-N-SH cells.

The channel blockers were added to each vial in the presence of MPTP when initiating experiments. Cell viability was assayed after 96hr culture.

*, $p<0.05$, compared with the control group. (n=5)

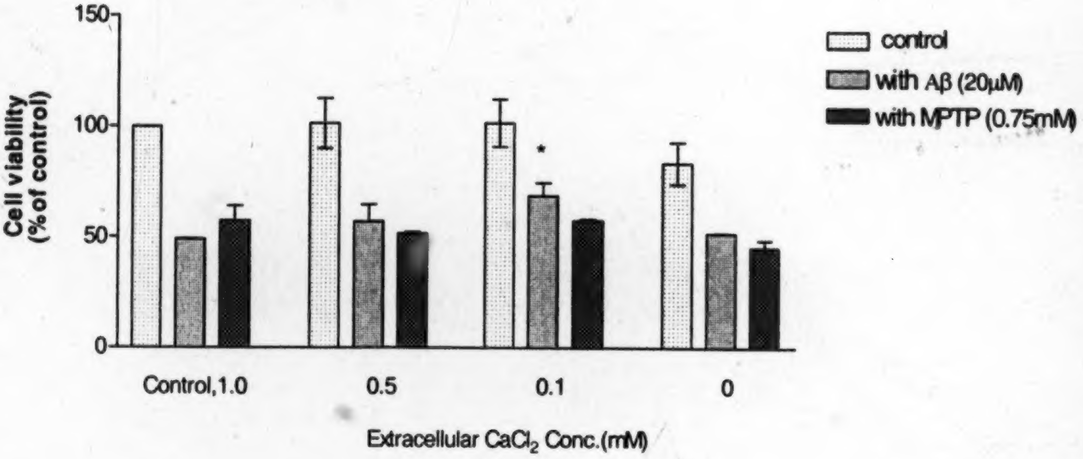


Fig. 5.3. The effects of extracellular Ca^{++} concentrations on Aβ (20μM)- and MPTP (0.75mM)-treated SK-N-SH cells.

Cell culture media were modified to contain 0, 0.1, 0.5mM $CaCl_2$, (1mM in control, with regular MEM medium). Cells were cultured with Aβ or MPTP when initiating experiments. Cell viability was assayed after 96hr.

*, $p<0.05$, compared with the Aβ group. (n=3).

*, $p<0.05$, when compared with control group. (n=5)

A:

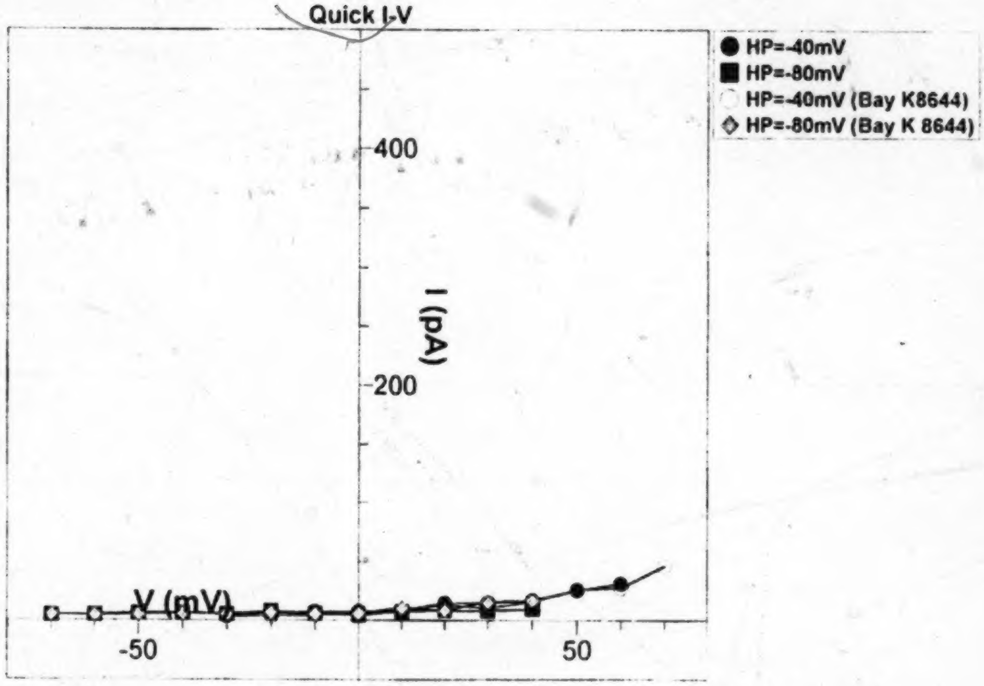
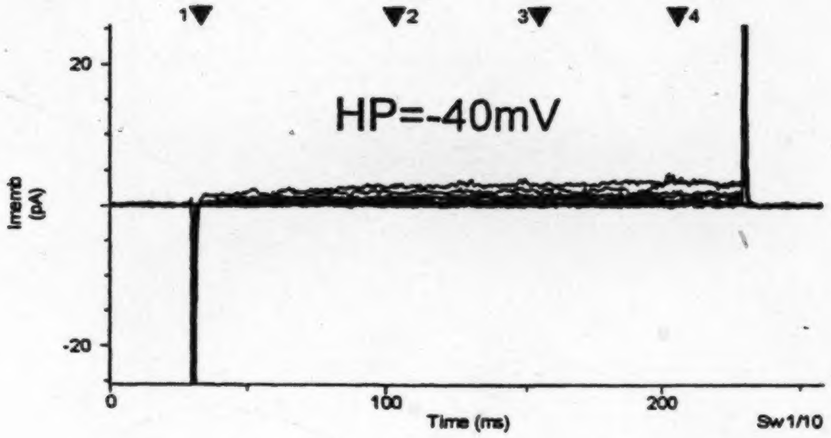


Fig. 5.4. The Ca^{++} channel recording of SK-N-SH cells with the whole-cell version of patch clamp technique.

A: Summarized current-voltage relationship for SK-N-SH cell with holding potentials of –40mV and –80mV (for L-type, and T-type activation), respectively, with or without the presence of Bay K8644 (10^{-6}M). B: No tracings were evoked with the holding potential at –40mV; the addition of Bay K8644 did not change the recordings. C: No tracings were evoked with the holding potential at –80mV; the addition of Bay K8644 did not change the recordings.

B: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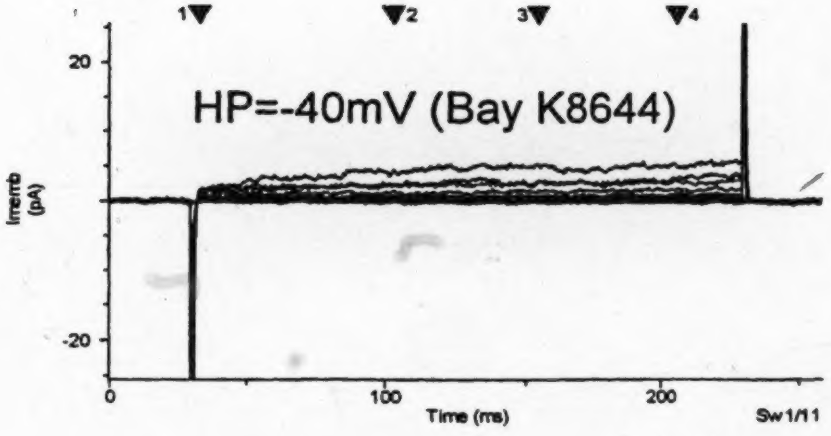
2
3
4
HP=-40mV
20
-20
Imemb (pA)
0
100
200
Time (ms)
Sw1/10


1
2
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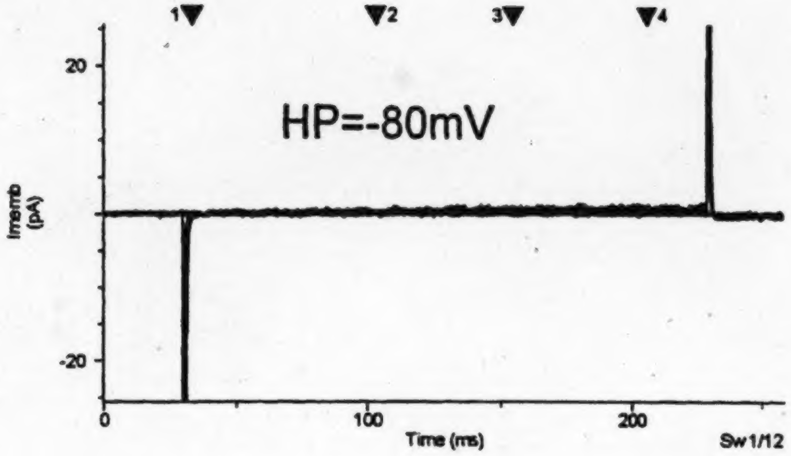
4
HP=-40mV (Bay K8644)
20
-20
Imemb (pA)
0
100
200
Time (ms)
Sw1/11

C: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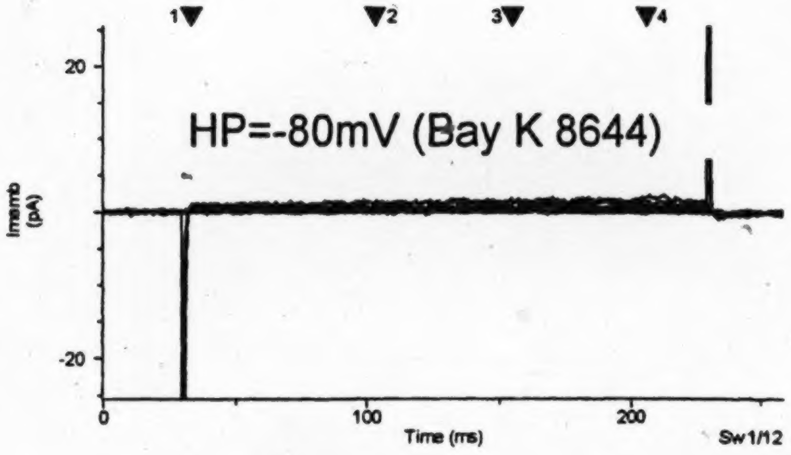
Imemb (pA)
-20
0
100
Time (ms)
200
Sw1/12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HP=-80mV (Bay K 8644)
Imemb (pA)
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Time (ms)
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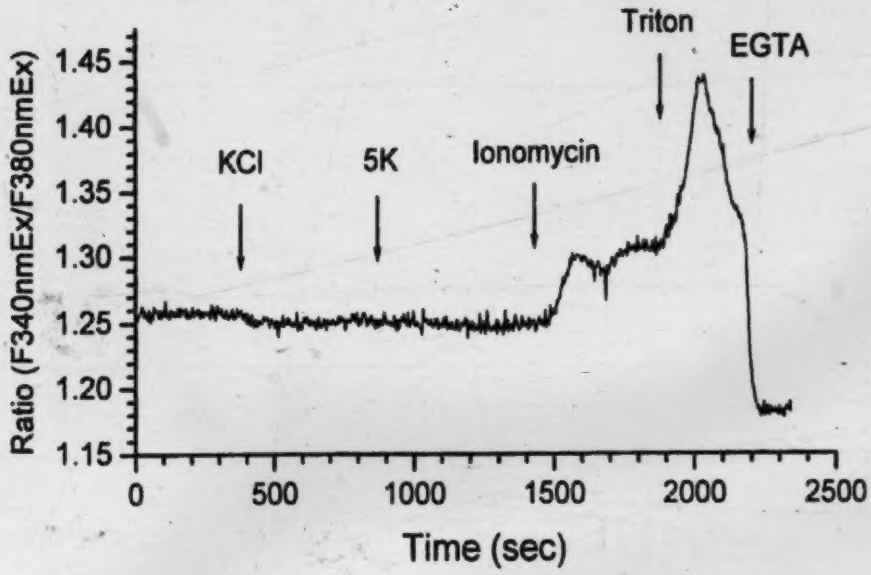
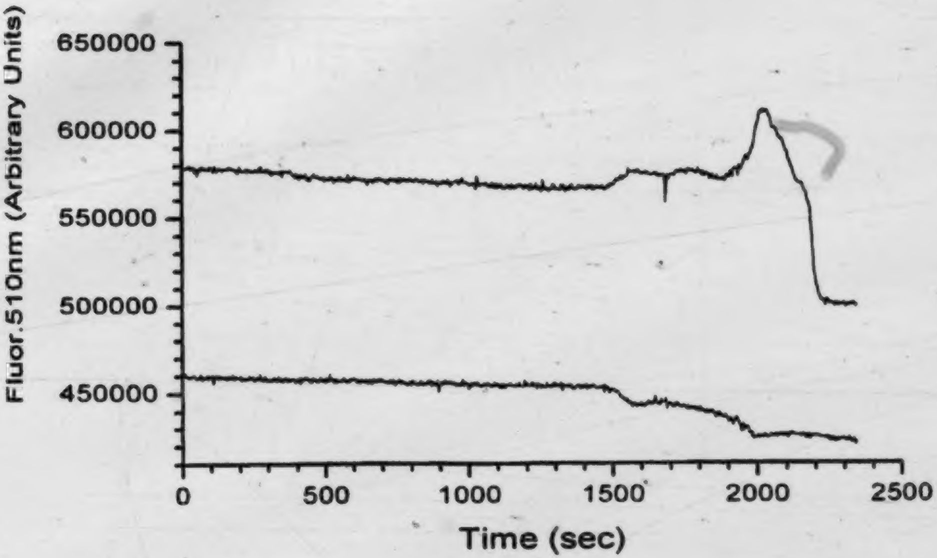
Sw1/12

A:


Ratio (F340nmEx/F380nmEx)
1.45
1.40
1.35
1.30
1.25
1.20
1.15
KCl
5K
Ionomycin
Triton
EGTA
0
500
1000
1500
2000
2500
Time (sec)

B:


Fluor.510nm (Arbitrary Units)
650000
600000
550000
500000
450000
0
500
1000
1500
2000
2500
Time (sec)

C:

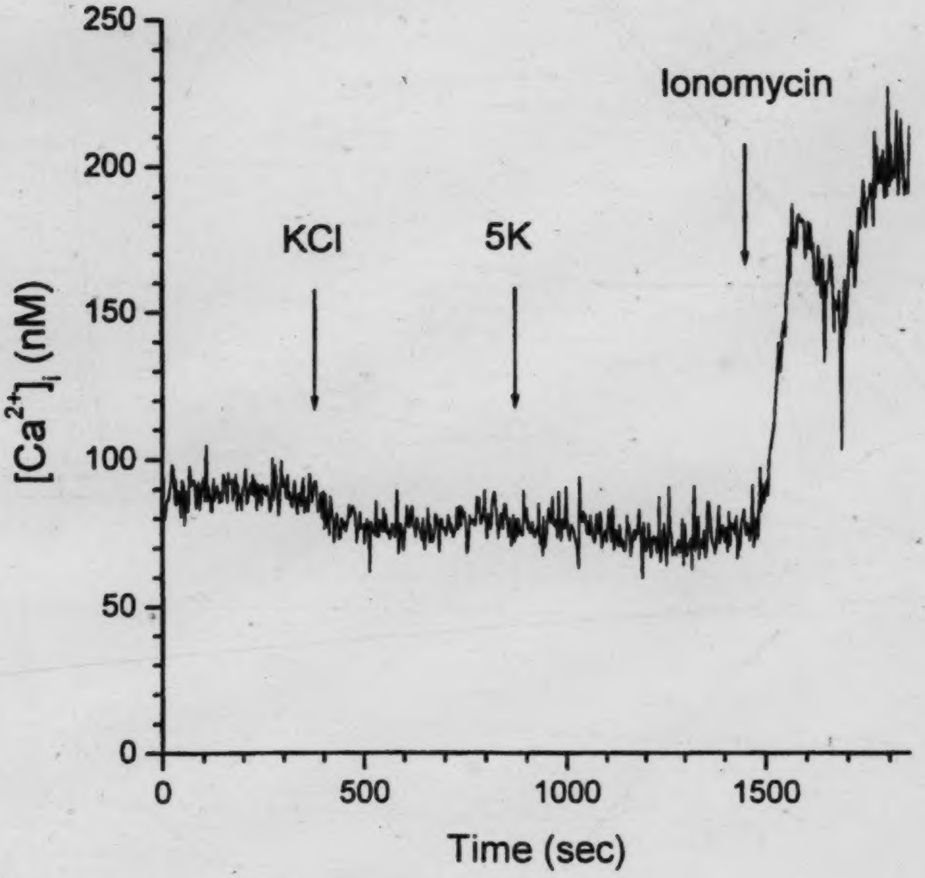


Fig. 5.5. The responses of SK-N-SH cells to 30mM KCl stimulated depolarization.

No $[Ca^{++}]_i$ increments were recorded as reflected by the ratio (A) and the arbitrary units with an emission wavelength of 510nm (B). $[Ca^{++}]_i$ remained constant with the application of 30mM KCl (C).

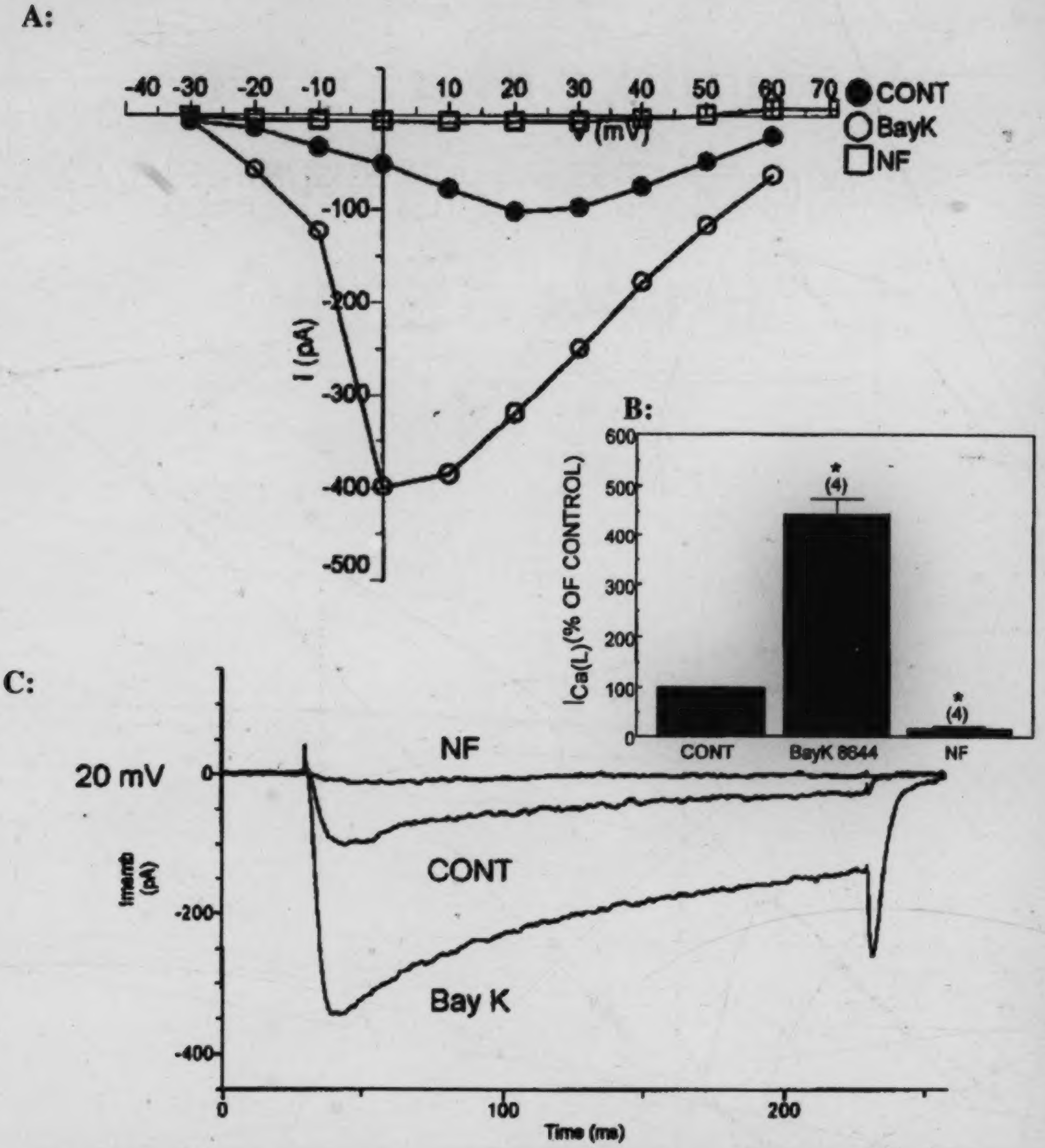
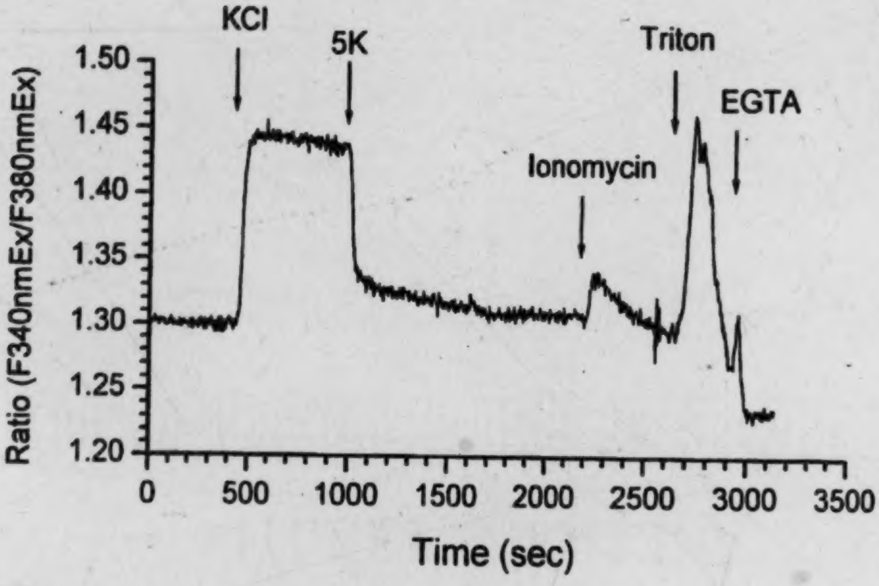


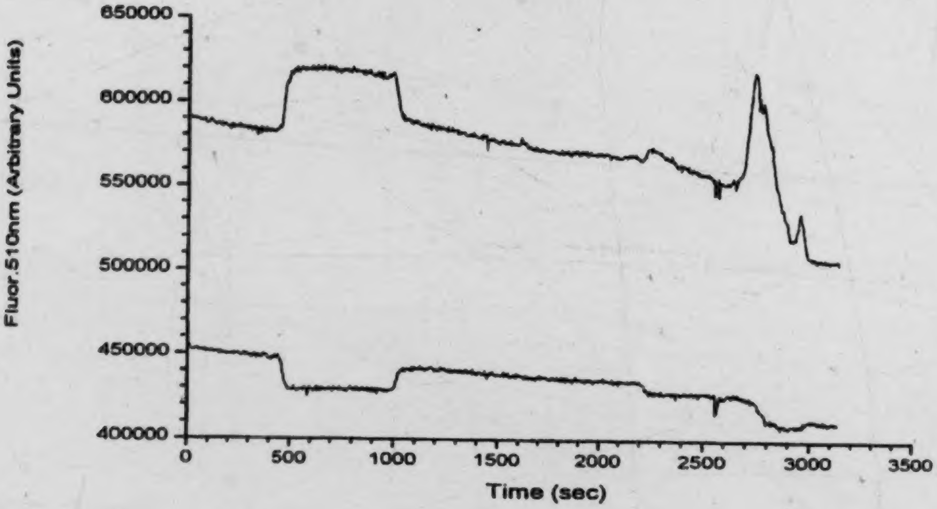
Fig. 5.6: The recordings of L-type VDCC in GH3 cells with the holding potential at -40mV.

A: Current-voltage relationships in control (•) and that obtained after application of Bay K8644 (10^{-6} M) (O) and nifedipine (10^{-6} M) (□). B: The effects of Bay K8644 and nifedipine on L-type VDCC currents evoked by depolarizing cells from a holding potential of –40mV. *, $p<0.05$, compared with control, n=4. C: Original records of Ca^{++} currents with the presence of Bay K8644, or nifedipine with a testing potential of 20mV.

A:


KCl
5K
Ionomycin
Triton
EGTA
Ratio (F340nmEx/F380nmEx)
1.50
1.45
1.40
1.35
1.30
1.25
1.20
0
500
1000
1500
2000
2500
3000
3500
Time (sec)

B:


Fluor.510nm (Arbitrary Units)
650000
600000
550000
500000
450000
400000
0
500
1000
1500
2000
2500
3000
3500
Time (sec)

C:

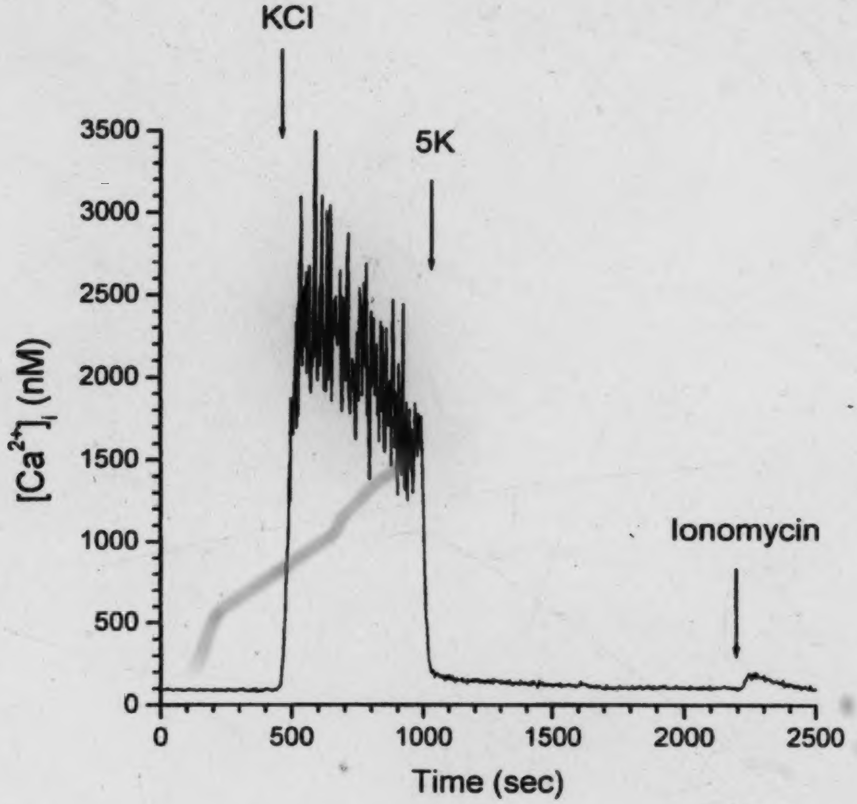
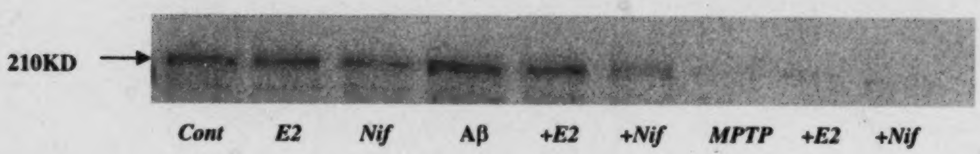


Fig. 5.7. The responses of GH3 cells to 30mM KCl stimulated depolarization.

Dramatic $[Ca^{++}]_i$ increments were recorded as reflected by the ratio (A) and the arbitrary units with an emission wavelength of 510nm (B). $[Ca^{++}]_i$ increased from 0.1μM to 2.36μM in response to the application of 30mM KCl (C), and the increment was removed after the bathing solution switched to 5K solution.

A: **The expression of L-type Ca^{++} channel DHP binding complex (α_1 subunit) in Aβ- and MPTP-treated SK-N-SH cell culture**



B:

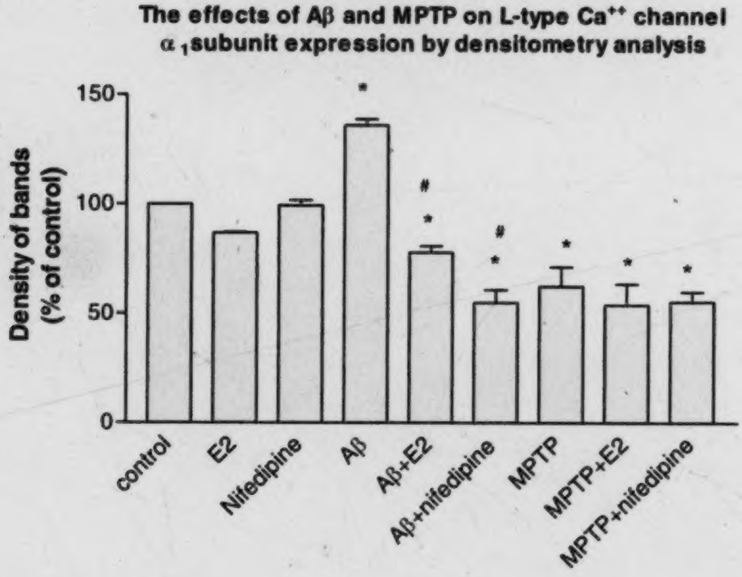

The effects of Aβ and MPTP on L-type Ca^{++} channel α_1 subunit expression by densitometry analysis

Fig. 5.8. The effects of Aβ and MPTP on the expression of α_1 subunit (DHP binding complex) of L-type VDCC in SK-N-SH cells.

The expression of α_1 was investigated with the addition of E2 (0.1n M) or nifedipine (5μM) by Western blotting with anti-dihydropyridine binding complex (α_1 subunit). A: Western blot showing the expression of α_1 subunit in each treatment group.
B: Densitometry analysis of the protein expression in each group.

*, $p<0.05$, compared with the control group;
#, $p<0.05$, compared with the Aβ group. (n=4)

A: **The expression of L-type Ca^{++} channel α_{1C} subunit in Aβ- and MPTP-treated SK-N-SH cell culture**

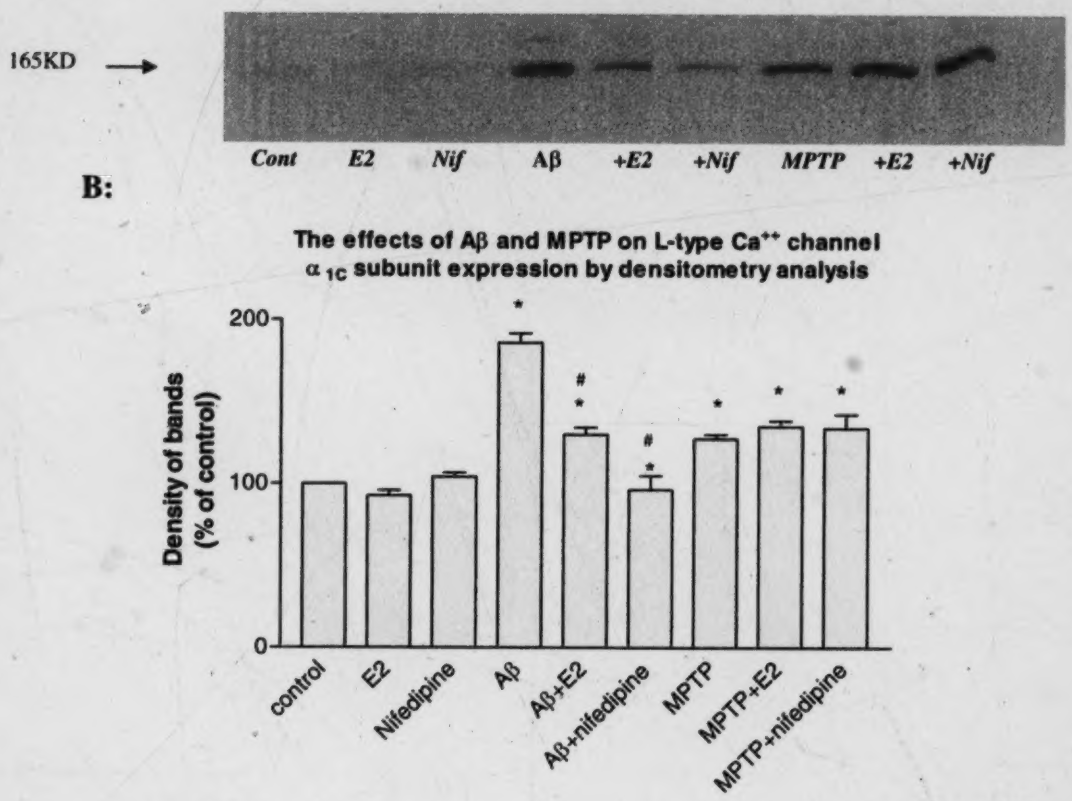
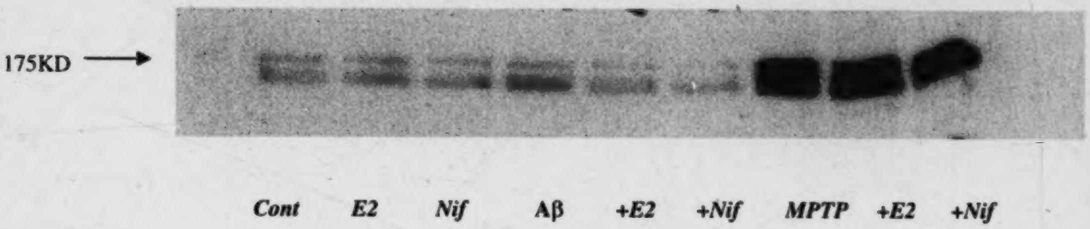


Fig. 5.9. The effects of Aβ and MPTP on the expression of α_{1C} subunit of L-type VDCC in SK-N-SH cells.

The expression of α_{1C} was investigated with the addition of E2 (0.1nM) or nifedipine (5μM) by Western blotting with anti-α_{1C} antibody.
A: Western blot showing the expression of α_{1C} subunit in each treatment group.
B: Densitometry analysis of the protein expression in each group.

*, $p<0.05$, compared with the control group;
#, $p<0.05$, compared with the Aβ group. (n=3)

A: **The expression of L-type Ca^{++} channel α_{1D} subunit in Aβ- and MPTP-treated SK-N-SH cell culture**



B:

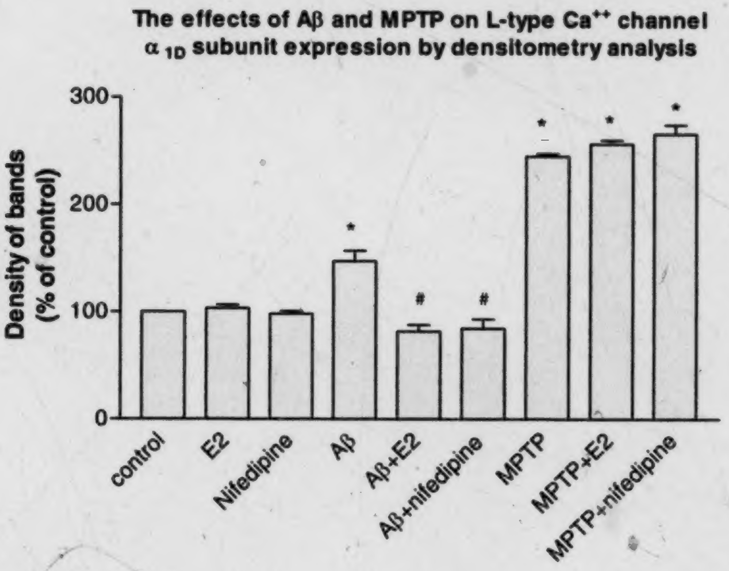


Fig. 5.10. The effects of Aβ and MPTP on the expression of α_{1D} subunit of L-type VDCC in SK-N-SH cells.

The expression of α_{1D} was investigated with the addition of E2 (0.1nM) or nifedipine (5μM) by Western blotting with anti-α_{1D} antibody.
A: Western blot showing the expression of α_{1D} subunit in each treatment group.
B: Densitometry analysis of the protein expression in each group.

*, $p<0.05$, compared with the control group;
#, $p<0.05$, compared with the Aβ group. (n=3)

Chapter 6

General Discussion and Conclusion

6.1. General discussion

The objective of the study described in this thesis was to investigate the protective effects of estrogen in several cytotoxic models, especially in neurodegenerative related cytotoxicities. Estrogen has been shown to act as a neuroprotectant. It may play a protective role in several ways. Estrogens have been reported to decrease the risk of neurodegenerative diseases and attenuate injury by suppressing the effects of neurotoxic or ischemic stimuli or by increasing the resilience of the brain to injury. [154, 223, 505, 516] Several mechanisms may account for these effects. Estrogens selectively enhance the growth and differentiation of axons and dendrites (neurites) in the developing brain, an effect that may be recapitulated following injury later in life. [212, 563] Estrogens also exert anti-apoptotic effects on neuronal cells that are mediated by activation of MAP kinases [53] or PKA [241, 464] and PKC, [242, 440] down-regulation of the expression of neurotrophin receptors, or by altering free radical production or free radical action on cells. [379, 507] Estrogens may also regulate the cholinergic system by inducing AchE and its activity or the receptor for NGF, by reducing the tone of dopaminergic neurons, and by antinociceptive and analgesic actions. [102, 223, 560] Among the large array of protective actions, the function of estrogen in cell survival plays a fundamental role in neurodegenerative changes and brain injuries. Some of the mechanisms underlying these effects are independent of the classically defined nuclear ERs and involve membrane receptors, direct modulation of neurotransmitter receptor function, or the known antioxidant activities of estrogen. [21, 39] Other neuroprotective effects of estrogen do depend on the classical nuclear ERs, through which estrogen alters expression of estrogen responsive genes that play a role in apoptosis, axonal regeneration, or general trophic support. Yet another possibility is that ERs in the membrane or cytoplasm alter phosphorylation cascades through direct interactions with protein kinases or that ER signaling may converge with signaling by other trophic molecules to confer resistance to injury. The regulatory effect of E2 in cellular calcium homeostasis accounts for its neuroprotective actions such as anti-apoptosis and anti-oxidation. The current studies concentrated on the effects of estrogen on cell survival. In particular, the cellular mechanisms relating to the regulation of calcium function were investigated.

Although many reports have demonstrated the neuroprotective effects of estrogen in a variety of neuronal tissues in relation to several different cellular mechanisms, many problems exist in these studies.

1). Studies of similar designs yield conflicting results when performed in different neuronal cell lines or preparations;

2). Most work on the neuroprotective actions of estrogen has been focused on the anti-apoptotic cascades, and evidence concerning anti-necrotic pathways is limited;

3). Cellular calcium homeostasis disturbance has been hypothesized to occur in neurodegenerative changes. The neuroprotective effects of estrogen have been considered to stabilize calcium homeostasis. [228] However, a direct link between estrogen effects and calcium homeostasis was lacking.

Therefore, the current studies were designed to illustrate the neuroprotective effects of estrogen in both apoptotic and necrotic cytotoxic models. The experiments were constructed to investigate how estrogen exerts neuroprotection through restoration of cellular calcium dysregulation. However, in addition to ascertaining how estrogen produced neuroprotection, there have been other interesting findings from these studies.

For instance, the establishment of a cytotoxic model system itself potentially has great significance in neuronal cytotoxic and protective research. Moreover, Ca^{++} channel blockers were used in a study to reveal how Ca^{++} influx through VDCCs was modified by cytotoxic insults. The results from these studies indicated that L-type channel blockers had additional features to protect against neuronal death through mechanisms other than blockade of Ca^{++} entry through VDCCs. The findings of the current studies are summarized in three sections as follows:

1). The significance of the established cytotoxicity models in a specific cell line, the SK-N-SH neuronal cell line;

2). The neuroprotective functions of E2, in apoptotic or necrotic models, with or without the mediation of ERs;

3). Involvement of the regulation of calcium homeostasis by E2 and the L-type VDCC blocker, nifedipine in neurodegenerative changes.

6.1.1. Four cytotoxic models were characterized, and are physiologically significant models for AD, PD, trophic factor withdrawal induced apoptosis, and ischemic research

A reliable *in vitro* cytotoxic system is essential in neurocytotoxic and neuroprotective research. Neuronal injuries and neuroprotection are of great interest in neuroscience research. Development of neuroprotective therapies for injuries or disease, such as stroke or neurodegenerative diseases, is dependent on defining the precise mechanisms whereby molecules or toxins are able to induce relatively selective injury of specific neurons. The present study utilized 4 cytotoxic models with the SK-N-SH human neuroblastoma cell line; these were Aβ, MPTP, high density, and serum deprivation induced neuronal death.

Aβ has been of great interest in AD research. Aβ deposition is a hallmark in the brains of AD patients. Aβ is a 4Kda protein which forms extracellular aggregates, and leads to neuronal death. Our results showed that Aβ 25~35, the toxic portion of the Aβ molecule, induced significant cell death in SK-N-SH culture at a concentration of 20μM after 96hr culture. Results with ELISA qualification of mono- and oligonuclesomes and with DNA laddering gel demonstrated that Aβ-induced neuronal death in SK-N-SH cells did not involve apoptotic pathways. Therefore, the mechanism presumably involves necrotic pathways. However, Aβ induced apoptosis has been observed in several other studies with primary cortical [21, 596] and hippocampal cultures, [13, 298, 588] and in several cell lines. [130, 299, 487] On the other hand, a necrotic type of neuronal death induced by Aβ has been reported in PC12 cells and rat cortical neurons. [38, 120, 326, 546] It has also been concluded from many reports that apoptotic neuronal death induced by Aβ was cell type dependent. [240] Moreover, in the present study, the addition of the MAP kinase inhibitor, SB203580 (1μM), significantly inhibited cell death by Aβ, suggesting the cytotoxicity of Aβ in SK-N-SH cells occurred at least partially through disruption of the Ras/Raf, MAPK cascade.

The discovery of MPTP, a dopaminergic toxin, has been extremely important in providing a model in PD research, since the exogenous toxic substance can initiate a PD-like syndrome. Our experiments have shown that MPTP (0.75mM) induced apoptosis in SK-N-SH cells, as revealed by mono- and oilgonucleosome qualification, and DNA laddering. Apoptosis is a well programmed process, involving gene regulation and protein synthesis. A protein synthesis inhibitor, cycloheximide, significantly restored the cell viability loss in the MPTP model. This further illustrated that MPTP induced apoptosis in the culture. MPTP and its downstream product, MPP^+, have been shown to produce apoptotic cell death in nigral dopaminergic neurons, [152, 153, 262, 472] and in several cell lines. [143, 163, 214, 342] In addition, the toxicity of MPTP and MPP^+ are involved in the inhibition of mitochondrial respiration, mitochondrial energy deprivation, [30, 456, 472] and accumulation of toxic ROS. [97, 302, 514] Therefore, neuroprotective candidates with antioxidant and anti-apoptotic properties can be seriously considered in PD therapeutic plans.

Serum deprivation is a well established model in neuronal cytotoxic and apoptotic related signal transduction research. We have found that both complete (0% FBS) and partial (1 or 3% FBS) removal of serum from culture media, which represent trophic factor and nutrient withdrawal, could induce apoptotic death in SK-N-SH cell culture after 96hr. Serum deprivation-induced neuronal apoptosis has been observed in a variety of model systems, including mouse cerebellar granule neurons, hippocampal neurons of rat embryo, [17, 630] rat cortical neurons, [249, 261] and SH-SY5Y cells. [97] Serum deprivation-induced apoptosis is usually mediated through growth factor withdrawal. In general, growth factor withdrawal activates JNK, and phosphorylates *c-Jun*, which in turn induces the activation of *Bax*, causing mitochondrial damage, which results in the release of cytochrome *c*, and activates the downstream caspase-9/3 pathways, resulting in apoptosis. A lack of trophic factor signaling also induces a non-nuclear competence-to-die pathway that facilitates the formation of the cytochrome *c*/Apaf-1/caspase-9 complex, resulting in caspase- activation. [272] Research has shown that the addition of several growth factors, including NGF,[286, 543] FGF,[142] and HGF[630] exerted neuroprotective effects in serum deprivation induced apoptosis in PC12 cells, retinal neurons, rat cerebellar granule neurons, and others.

Another cytotoxic model investigated in the present studies was a high density culture model, which may mimic the condition in ischemic injuries. A high density of cells plated without increasing the media volume placed the cells in an unfavorable condition, with the lack of nutrients, growth factors, and even adhesive surface. Apoptotic cell death has been observed in the high density culture model. The presence of the PKC inhibitor, chelerythrine, was able to reverse the viability loss to some extent. Although the precise mechanisms of the high density model have not been fully understood, we suspect this model mimics conditions of ischemic brain injury by virtue of nutrient and energy depletion, and oxygen insufficiency. Therefore, this model may be of great significance in stroke related research. The observation of significant cell viability loss, apoptotic characteristics, and the involvement of signal transduction suggested that this model may be related to oxidative stress and mitochondrial dysfunction.

The goals of neurotoxicity research are to identify molecular and cellular mechanisms that underlie neuronal functions under pathological conditions, and to explore adequate and suitable therapeutic strategies for different neuronal diseases and brain injuries. Neurotoxicity research is the basis of neuroprotection studies. Cell and tissue cultures are practical tools with which to pursue these goals. *In vitro* studies offer advantages over *in vivo* methods such as defined cell types, an extracellular environment that can be precisely manipulated, and direct observation. In the current studies, we have investigated reliable cytotoxic systems in which multiple insults induced both apoptotic and necrotic neuronal death in the SK-N-SH cell line. Since SK-N-SH cells have a specific neuronal phenotype and are responsive to various neuronal toxins and insults; thus they may be very useful in the assessment of neurotoxic and neuroprotective studies. Current results of studies with Aβ-, MPTP-, and serum deprivation-induced cell death correlate with some of the previous observations, [214, 224, 225, 227, 229, 472, 527] and the observed apoptotic characteristics in the MPTP and serum deprivation models in our studies also confirmed previous reports. [5, 143, 172, 214, 472] Although the cellular mechanisms are not as well understood, the high density model showed some similarities to ischemic brain injuries.

The available *in vitro* cytotoxic studies have provided abundant information, but much of that is conflicting. Some examples are that Aβ induced apoptosis is cell type dependent, [240] and MAPK inhibitors can protect against MPTP induced neuronal death [133] in mouse N2a neuroblastoma cells, but not in SK-N-SH cells. The differences among these experiments are very likely due to the differences of the cell lines and the systems tested since each cell line will respond to different insults through its unique signal transduction pathways. The present cytotoxic systems studied are of great physiological and pathological significance because they allow the study of neurotoxicity and "neuroprotection" under conditions in which the different pathways and mechanisms of neurons can be considered within one cell line and system. Hopefully, this approach can avoid variations which may be due to differences in the systems studied. Moreover, the current cytotoxic models involve both apoptotic and necrotic neuronal death, and each model is uniquely linked to different signal transduction pathways as revealed by the results with different kinase inhibitions. The multiple cascades of signal transduction pathways may represent important specific targets for neurotoxic compounds. Additionally, the tested cytotoxic models are relevant to oxidative stress, ROS accumulation, mitochondrial dysfunction and calcium homeostasis disruption, the common causes of neuronal cell death in most pathological conditions. Finally, these models have a close clinical relevance. Aβ is a neurodegenerative insult in AD while MPTP administration induces Parkinsonism. The high density model can be considered an *in vitro* model for brain ischemia. These are all key topics in neurotoxicity research. Any potential neuroprotective candidate with promising protective effects in any model will be a useful treatment option in related neurodegenerative diseases or brain injury.

6.1.2. E2 exhibits neuroprotective properties in both apoptotic and necrotic models

During the past decade, our understanding of estrogen as a potent protective factor in many physiological systems has increased remarkably. It has been demonstrated that, in addition to its effects on the reproductive axis and reproductive target organs, estrogen influences numerous nonreproductive functions such as bone and mineral metabolism,[18, 58, 59] cardiac and vascular function,[151, 349, 461] memory, cognition and mood,[178, 179, 519] and the incidence and progression of age-related diseases.[157, 176, 465] Moreover, the discovery of the ERs has led us to reevaluate new potential targets and diverse mechanisms of estrogen actions that have not been considered previously. An accumulating body of evidence clearly indicates that estrogen is a potent neuroprotective and neurotrophic factor: it influences memory and cognition, decreases the risk of and delays the onset of neurological diseases such as AD, and attenuates the extent of cell death that results from brain injuries such as cerebrovascular stroke and neurotrauma. [60, 154, 194, 228, 527] Thus, estradiol appears to act at two levels: 1) it decreases the risk of disease or injury; and/or 2) it decreases the extent of injury incurred by suppressing the neurotoxic stimulus itself or increasing the resilience of the brain to a given injury. [605] Clearly, it is critical for us to understand the cellular and molecular mechanisms that underlie the neuroprotective actions of estrogen.

Our findings showed that E2 acted as a neuroprotectant in cell death induced by multiple insults, including Aβ, MPTP, serum deprivation or low serum culture, and a nutrient deprived high density culture model. E2, in a relatively broad range of concentrations, prevented cell viability losses in both apoptotic and necrotic cell death in a bi-phasic manner. In all four models, the physiologically relevant concentration of E2 (at around 0.1nM) had significant protective effects. The observation that E2 was capable of preventing cell death induced by Aβ and MPTP at physiologically relevant concentrations supported the hypothesis that ERT was somewhat beneficial in AD and PD patients. E2 at physiological concentrations may play a fundamental role in the protection of neurons from different insults, including the pathological changes involved in AD and PD. The effects of E2 in the high density model suggested the possibility that ERT may prevent the effects of stroke, or that E2 can be part of the treatment plan in brain ischemia. The ability of E2 to prevent neuronal death induced by Aβ and serum deprivation has been reported previously. [36, 42, 213, 224, 225, 228, 313] The direct cytoprotective effect of estrogen against MPTP is not well understood. Our results clearly demonstrated that E2 had an anti-cytotoxic effect against neuronal death induced by all four insults in the SK-N-SH cells. Concerning the effects of E2 in the four models, our results were the first to indicate that physiological concentrations of E2 were capable of protecting against the cell death induced by the dopaminergic toxin, MPTP, *in vitro* with neurons other than primary nigral dopaminergic neurons. This model has great potential for *in vitro* studies of PD related neuronal loss, and it is ideal for investigating potential neuroprotective candidates.

The anti-apoptotic effects of E2 have been reported in many studies. It can protect against neuronal apoptosis by stabilizing calcium homeostasis, reducing ROS formation, regulating caspase activities, maintaining mitochondrial membrane integrity, and stimulating expression of several anti-apoptotic proteins. Several studies suggest that estrogen regulates *bcl-2* expression in various tissues, including brain. [488] Dubal *et al.* [155] showed that E2 treatment in ischemic brain significantly increased *bcl-2* levels and enhanced neuronal survival. The observations in the present studies with MPTP, serum deprivation and high density culture have supported the anti-apoptotic actions, since E2 was neuroprotective in several models which involved apoptosis. Additionally, when assayed after 96hr exposure to Aβ, apoptosis could not be detected. Thus, the cell death in the Aβ model was presumably through necrosis. The ability of E2 to restore Aβ induced cell viability loss was presumably mainly through anti-necrotic pathways, and E2 was capable of inducing very strong and effective neuroprotection against the apparent necrosis.

When comparing the efficacy of the neuroprotective actions of E2 in all four cytotoxic models, it was noticed that E2 (0.1nM) almost fully restored the cell viability loss in the Aβ model, while E2 at the same concentration was only partially protective in the other three apoptotic groups. These results suggested that E2 had a greater capacity to prevent necrotic type cell death than apoptotic cell death in the SK-N-SH cell line after a 96hr culture period. Although apoptosis has been intensely investigated and it has been noticed that a close correlation exists between apoptosis and several degenerative diseases and injuries of the brain, necrotic type death can not be ignored. Nerve cell death is the central feature of human neurodegenerative diseases. Apoptosis and necrosis may overlap, occur sequentially under certain conditions, and may not be detected unequivocally. For instance, metabolic defects leading to ATP depletion can preclude caspase activation and consequently switch execution of cell death towards necrosis. Following excitotoxic injury of the rat striatum, it has been shown that internucleosomal DNA fragmentation (evidence of apoptosis) was seen at early time intervals and random DNA fragmentation (evidence of necrosis) at later time points. [441] The observation that E2 was capable of protecting against Aβ induced necrosis is an important line of evidence in neurodegeneration and brain injuries.

6.1.3. The neuroprotection of E2 may occur via or independent of ER

It has become increasingly clear that estrogen exerts neuroprotective effects via traditional ER-dependent and novel ER-independent mechanisms of action. The predominant mechanisms may depend upon the type of neuronal injury, and/or the dose of hormone administered. In general, it appears that physiological levels of estrogen protect via mechanisms involving ERs and changes in gene expression. The interactions of estrogen with its receptors may lead to the expected classic downstream events: receptor dimerization, receptor binding to estrogen response elements on DNA, and induction of transcription of target genes. Alternatively, interactions with receptors may also elicit novel cross-talk with second messenger molecules that lead to phosphorylation and activation of key proteins, such as certain growth factors and their receptors. In contrast, pharmacological levels of estrogen appear to by-pass ERs and invoke mechanisms that involve antioxidant actions, and/or NO production. However, a more recent report by Green and Simpkin [228] showed that the presence of glutathione in cell cultures dramatically reduces the effective concentration at which estrogen exerts protective effects *in vitro* under circumstances where receptors were not required. Thus, under some circumstances, low levels of estrogen may protect via receptor-independent mechanisms. In the current studies, we have demonstrated that neither tamoxifen nor ICI 182,780 could abolish E2 effects on Aβ, trophic factor withdrawal or high density cultures. Therefore, it can be postulated that in a 96hr culture period, novel ER mediated protective actions were not the primary mechanism of the observed neuroprotection. Mechanisms related to anti-oxidation, attenuated ROS accumulation, or stabilizing calcium homeostasis disruption might be important in these models. Moreover, exciting new evidence suggests that estrogen may protect against injury via receptor-dependent or receptor-independent mechanisms that involve cross-talk with other second messenger signaling molecules such as cAMP, MAP kinases, or molecules of the PI-3K/Akt pathway. [605] These mechanisms may allow estrogen to act rapidly through phosphorylation and activation of preexisting critical proteins. Estrogen can also act after some delay through phosphorylation-dependent genomic actions. The detected effects of kinase inhibitors in the Aβ and high density models may support this notion. Further work is necessary to explore the link between E2 protection and protein phosphorylation in these models.

In SK-N-SH cells, only ERβ, not ERα, is expressed as detected with the Western blot technique. Studies performed both *in vivo* and *in vitro* suggested that physiological concentrations of E2 protected through ER-dependent mechanisms that led to transcription of critical genes that ultimately promoted cell survival. [606] The observation that tamoxifen partially, and ICI 182,780 completely, antagonized the protective effects of E2 in the MPTP model suggested that the E2 protection in this model was through the ERβ mediated pathway. The lower efficacy of tamoxifen in the blockade of E2 effects may have been due to its SERM characteristics, in which tamoxifen may be a partial ER agonist in certain tissues. ICI 182,780 is considered to be a pure ERβ antagonist with a higher affinity to ER, although a few studies suggested ICI 182,780 can also act as a partial ERα agonist. [572]

It is well accepted that estrogen influences the expression of numerous genes that are relevant to this hormone's ability to protect. For example, estrogen affects, in complex ways, the expression of genes that are involved in the balance of apoptosis and cell survival, [194] mitochondrial function, [379, 385] synthesis and secretion of neurotransmitters that modulate neuronal excitability or neuron/astrocyte interactions, [179, 203, 403] expression of neurotrophins, growth factors, and their receptors leading to enhanced neuronal viability, [49, 100, 208, 210, 212, 223] and expression of factors that influence dendritic or axonal elongation [533] and synaptogenesis. [426] The current observation that E2 protected against MPTP induced apoptotic neuronal death through ERβ mediated pathways provided more direct evidence supporting the involvement of ER in cell survival in this model.

The finding in current study demonstrated that physiologically relevant concentrations of E2 show more profound protective effects in all four models. This suggests that the circulating level of estrogen in premenopausal women might play a fundamental role in maintaining several aspects of neuronal function. The present studies have also demonstrated that the effects of E2 can be dependent or independent of the activation of ER pathways, and the dependency of ER is insult dependent, and is not determined by the characteristics of the cell death. These are the first clear demonstrations that the ER-dependency in neuroprotective actions may even vary within one cell line, and the requirement for ER activation may be determined by the injury type, rather than the cell death type.

6.1.4. Effects of E2 and the L-type VDCC blocker, nifedipine, supported the involvement of calcium homeostasis regulation in neurodegenerative changes

$[Ca^{++}]_i$ is of great importance in cellular functions. For example, ionic conductance of Ca^{++} in neurons is essential due to its role in the control of cell excitability and synaptic transmission. Ca^{++} is involved in both cell survival and cell death, and it acts as a second massager in a large variety of biological responses. VDCCs, among all the mechanisms involved in cellular calcium homeostasis, are of particular functional importance, since they are related to electrical signaling and in coupling electrical signals to changes in cytoplasmic calcium. The Ca^{++} influx through neuronal VDCCs regulates cellular processes including neurotransmitter release, activation of Ca^{++}-dependent enzymes and second messenger cascades, gene regulation, and cell proliferation. [535]

It has been suggested that altered Ca^{++} homeostasis might be the fundamental mediator of age-related changes in the nervous system. [377, 389] The available literature has indicated that estrogen is capable of regulating VDCCs. In the nervous system, Ca^{++} plays a key role in neuronal survival. The regulation of calcium homeostasis could be an important aspect of the neuroprotective actions of estrogen in neurodegenerative disorders and other brain injuries.

The present studies revealed that the L-type VDCC blocker, nifedipine (5μM), but not N, or T-type blockers, almost fully prevented the cell viability loss caused by Aβ. In addition, a decrease in extracellular calcium concentration (0.1mM $CaCl_2$, instead of 1.0mM $CaCl_2$) significantly protected against neuronal death in Aβ treated cells. These data suggested that Ca^{++} influxes through L-type VDCCs from the extracellular calcium pool, but not through other channels, might contribute to Aβ induced neuronal injury in SK-N-SH cells. Nifedipine, by attenuating such influxes, was able to attenuate Aβ toxicity. In the MPTP model, none of the VDCC blockers tested exhibited any protective effects, nor did the modulation of extracellular [Ca^{++}] change cell viability. These data suggested that calcium homeostasis disruption was not the predominant cause of cell death induced by MPTP. However, calcium homeostasis disruption could possibly have occurred along the cell death cascades in this model. The role of the VDCCs in Aβ neurotoxicity was examined previously in rat cultured cortical and hippocampal neurons. Aβ-induced neurotoxicity was attenuated with the addition of nimodipine, whereas application of N or T-type blockers showed no effects. [590] More data with the patch clamp technique indicated that the Ca^{++} current density of Aβ-treated neurons was approximately twofold higher than that of control neurons.[16] Also, Aβ increased Ca^{++} uptake, which was sensitive to nimodipine.

The current study failed to record VDCC activity with the patch clamp technique, and failed to record changes in intracellular calcium measured with the fura-2 technique. This is likely because the two methods are not applicable to dead or dying cells. Our results with Western blot analysis revealed that the protein expression of the α_1 subunit of L-type VDCC has been altered with different toxic insults. SK-N-SH cells expressed low levels of α_1 (the DHP binding complex) in the basal condition; the 2 isoforms of the neuronal α_1 subtype, α_{1C} and α_{1D} were both expressed in this cell line. α_1, α_{1C} and α_{1D} expression in SK-N-SH cells were all increased upon exposure to Aβ (20μM), and both E2 and nifedipine significantly reversed the overexpression. In contrast, MPTP treatment decreased the production of α_1 protein, but increased α_{1C} and α_{1D} expression. However, the addition of E2 or nifedipine did not modify any changes in the MPTP model. These observations were highly correlated with results obtained in cell viability studies with Ca^{++} channel blockers. In that case, nifedipine was able to protect SK-N-SH cells from Aβ induced cytotoxicity, but not MPTP induced cytotoxicity. Combined with the observations that neither E2 nor nifedipine changed the protein expression in the MPTP model and that nifedipine did not change the cell viability in the same model, it may be that the observed overexpression of these two isoforms in MPTP treated culture was probably due to non-specific increased protein expression associated with apoptotic cell death, and may also related to a nifedipine insensitive mechanism. The decrease in α_1

(DHP binding complex) protein expression in the MPTP model might suggest a decrement of cellular function and activity with the presence of the dopaminergic toxin.

There is growing evidence that Aβ is involved in the etiology of AD with altered calcium homeostasis. Several theories exist as to how Aβ contributes to the disruption of calcium homeostasis. Aβ1~40 has been proposed to form calcium channels in synthetic bilayer membranes. [468] It has also been reported that Aβ could act as an ionophore in a biological membrane. [27, 376] The ionophore action of Aβ may lead to an increase in $[Ca^{++}]_i$, and it is reported to be related to decrease in energy charge. Mark *et al.* [364] reported that exposure of cultured rat hippocampal neurons to Aβ 1~40 or Aβ 25~35 caused a selective reduction in Na^+/K^+-ATPase activity which preceded loss of calcium homeostasis and cell degeneration. They also demonstrated that impairment of this enzyme was sufficient to induce an elevation of $[Ca^{++}]_i$ and neuronal injury. Impairment of Na^+/K^+-ATPase activity appeared to be causally involved in the elevation of $[Ca^{++}]_i$ and neurotoxicity since suppression of Na^+ influx significantly reduced Aβ-induced $[Ca^{++}]_i$ elevation and neuronal death. In the current studies, we demonstrated that Aβ could cause an increased expression of L-type VDCCs. The elevated Ca^{++} influx perhaps occur through the newly synthesized and preexisting L channels, which then contribute to a dysregulation of cellular calcium homeostasis, leading to cell death.

E2 has been observed to exert protective effects in Aβ induced toxicity by regulating cellular calcium. Our results indicated that E2 was capable of restoring the Aβ induced overexpression of the α_1 subunit, in a magnitude similar to that produced by nifedipine. The protection by E2 in the Aβ model occurred in a manner similar to that of nifedipine in the cell viability study as well. This correlation suggested that in the Aβ model, the protective effects exerted by E2 and nifedipine were through stabilizing calcium homeostasis via maintaining the physiological level of the expression of L-type channels in the neuronal plasma membrane of SK-N-SH cells. Moreover, the protective effects of DHPs in the Aβ model could be postulated to occur through a genomic effect, by down-regulation of L-type channel protein expression rather than acute blockade of Ca^{++} entry. The effects of estrogen in regulating neuronal calcium homeostasis have been reported in several non-genomic studies. Mermelstein *et al.* [399] reported that physiological concentrations of E2 decreased Ba^{++} currents in neostriatal neurons through L-type channels via activation of a G-protein signaling pathway. Kim *et al.* [319] have observed that in PC12 cells, the actions of E2, which blunted 50mM KCl stimulated Ca^{++} influxes, were through the blockade of both N- and L-type VDCCs. Goodman *et al.* [217] reported that estrogens attenuated Aβ and glutamate-induced elevation in $[Ca^{++}]_i$. However, the mechanisms by which E2 affects neurodegenerative changes via Ca^{++} mediated pathways has not been clearly illustrated. The current observation that E2 protected against Aβ-induced neuronal cell death through a reversal of excessive L-type VDCC formation helped to further reveal the cascade of the neuroprotective actions of E2, and might be helpful for new therapeutic strategies in AD treatment.

Current studies revealed that calcium homeostasis disruption is a dominant factor in Aβ induced necrosis in SK-N-SH. E2 also protected against MPTP-induced apoptotic cell death, apparently through an ERβ mediated pathway, and it seemed that the VDCC abnormality was not the predominant factor in MPTP induced pathology. The protection against MPTP by E2 was probably due to anti-apoptotic and anti-oxidative properties of the steroid. The α_{1C} and α_{1D} subunits of the L-channel were overexpressed with the occurrence of apoptosis, suggesting some DHP insensitive channel formation was related to the programmed neuronal death process. The physiological relevance of the overexpression in L-type VDCC α_1 subunit when apoptosis occurred has not yet to be clarified.

Although there has been evidence demonstrating the effects of estrogen in protecting against Aβ cytotoxicity through regulating calcium homeostasis, the current study indicated for the first time that the protective actions were largely involved in the maintenance of an overexpression of the L-type VDCC proteins. Additionally, the results with the VDCC blockers showing that nifedipine protected neurons against Aβ cytotoxicity through restoration of the overexpression of L-type VDCCs suggesting that the protective action was through a genomic effect of this channel blocker. This is the first demonstration that DHPs are capable of regulating L-type VDCC protein expression when the protein expression is elevated under certain pathological conditions. The exact cascade in which nifedipine regulates protein expression is not clearly understood. It is hypothesized that nifedipine may cross the plasma membrane when the membrane permeability changes in the presence of Aβ. By binding to certain cytosolic or nuclear components, nifedipine may be able to modulate gene regulation and protein expression. This hypothesized genomic action of DHPs may help to better illustrate the previously observed neuroprotective actions related to some neurodegenerative changes and other brain injuries. A new fundamental role of DHPs will be considered in calcium homeostasis regulation.

6.2. Summary and future directions

6.2.1. Summaries from present studies

6.2.1.1. Establishment of a neuronal cytotoxic system

The current studies established a reliable *in vitro* neuronal cytotoxicity system with SK-N-SH human neuroblastoma cells. The characteristics of the cytotoxicity induced by each insult have been summarized in Table 4.1.

The current cytotoxic system is of great physiological and pathological significance because:

1). It allows the study of neurotoxicity and "neuroprotection" within one cell line and system, removing variations which may be due to different systems studied.

2). Many aspects of cytotoxicity are covered within one cell line. Both apoptosis and necrosis, and different signal transduction pathways and mechanisms of neuronal death can be investigated and compared.

3). The insults used in the present studies are of great clinical relevance. Aβ is an important neurodegenerative toxin in AD, and MPTP administration mimics Parkinsonism. The high density model represents several aspects in brain ischemic injuries. In general, besides its regulatory role in development, neuronal apoptosis is related to a large variety of neuronal diseases and brain injuries. Any neuroprotectants that exert protective effects in the current cytotoxic models can be considered for future therapeutic use.

6.2.1.2. Estrogen, a neuroprotectant against both apoptosis and necrosis

E2 was capable of protecting SK-N-SH cells from Aβ-induced necrosis, and from MPTP-, serum deprivation-, and high density-induced apoptosis. The observations suggested that E2 can protect against neuronal death through different pathways on multiple cellular levels. Additionally, the most effective concentrations of E2 were physiologically relevant concentrations, which supported the notion that physiological E2 in premenopausal women may play a fundamental role in maintaining a large array of neuronal functions. Moreover, it is demonstrated for the first time that E2 has a greater capacity to prevent necrotic, rather than apoptotic cell death in the SK-N-SH cell line after a 96hr culture period. This may lead to further exploration of the neuroprotective actions of E2 under different pathological conditions.

6.2.1.3. The neuroprotective actions of E2 can be either dependent on or independent of ER

In Aβ, serum deprivation, and high density models, the addition of antiestrogens, such as tamoxifen and ICI 182,780 failed to abolish the E2 effects, suggesting that the protection of E2 in these models was not mediated by traditional ER linked genomic pathways. The action of E2 may be non-specific, through antioxidant pathways, or attenuation of ROS accumulation. However, tamoxifen partially, and ICI 182,789 completely reversed the effects of E2 in the MPTP model, suggesting the protection exerted by E2 in the MPTP model was through ERβ mediated pathways.

It can be concluded that long term, genomic effects of E2 as a neuroprotectant can be either through traditional ER pathways or independent of ER, and the predominant mechanisms may depend upon the type of neural injury. These observations are the first to demonstrate that estrogen can either bypass or protect through the ER-mediated pathways in the same cell line. The anti-apoptotic or the anti-necrotic actions of estrogen may not be determined specifically by ER activation.

6.2.1.4. Calcium homeostasis and E2 effects

The mechanism of Aβ toxicity is unclear, but it is believed that the loss of calcium homeostasis plays an important role in inducing neuronal death. Results in this thesis have shown that:

1). The addition of the L-type VDCC blocker, nifedipine strongly protected SK-N-SH cells from Aβ induced cytotoxicity, suggesting blockade of Ca^{++} entry was protective in the Aβ model.

2). Decreasing the extracellular pool of Ca^{++} partially protected against neuronal death in the Aβ model, suggesting that excessive influx of Ca^{++} contributed to Aβ toxicity.

3). Analysis of the expression of L-type α_1 subunit showed that the calcium homeostasis disruption in the Aβ model may have been largely due to an overexpression of L-type VDCC. An increase in Ca^{++} influx through pre-existing and newly synthesized L-type channels may contribute to an elevation in $[Ca^{++}]_i$ leading to cell death.

4). Restoration of channel protein overexpression by E2, which is probably the cellular mechanism underlying the protective effects against Aβ-induced cytotoxicity in the SK-N-SH cells.

5). The traditional L-type channel blocker, nifedipine, also showed a profound protective effect against Aβ, probably also through the restoration of the L-channel overexpression. The blockade of Ca^{++} entry perhaps played a minor role in the protective process.

6). MPTP treatment changed L-type α_1 expression as well. However, the changes were not modified by either E2 or nifedipine, and the increment in α_{1C} and α_{1D} may have been related to apoptotic cell death.

7). The overexpression of α_{1C} and α_{1D} subunits of L-channel VDCC correlated with the occurrence of apoptosis, suggesting some non-specific elevated protein expression related to the programmed neuronal death process (see appendix) .

Within the systems investigated, there are not any data indicating direct effects of E2 on $[Ca^{++}]_i$ changes. However, collectively, the observed actions of E2 and nifedipine in the cell viability studies, and in the expression of the L-type VDCC proteins suggested that Aβ toxicity might be related to $[Ca^{++}]_i$ dysregulations.

6.2.2. New directions for research based upon the present studies

The current project is an extensive study in neurocytotoxicity and neuroprotection in one cell line, focusing on the effects of estrogen. It explores the regulation of cellular calcium homeostasis and mechanisms related to both apoptosis and necrosis. The cytotoxic models established in the present studies are of great pathophysiological and clinical significance, and the collective results may be applicable to therapeutic practice. Although the current studies have demonstrated several cellular mechanisms related to the neuroprotective effects of estrogen, they may not be conclusive. A few questions remain to be answered:

1). Estrogen exhibited protection in all four cytotoxic models. We have just revealed that among the four, Aβ induced necrosis after 96hr, while the other three insults induced apoptosis after 96hr. Each individual model induced cell death through unique signal transduction pathways. Protein phosphorylation may play some role in at least some of the models, since a MAP kinase inhibitor and a PKC inhibitor showed protection in Aβ and high density models, respectively. Whether and how estrogen can modify cell survival and death by regulation of protein phosphorylation should be considered in future studies.

2). The current studies demonstrated that E2 can exert protective effects in SK-N-SH cells either dependent or independent of ER mediation. The predominant mechanisms may depend upon the type of neural injury. It has been observed that ERβ, but not ERα was expressed in this cell line. Among the four cytotoxic models, E2 only protected MPTP treated cells through ER-mediated pathways. However, to further clearly demonstrate the "injury-type dependence" involvement in ER mediation, and to investigate how ERα affects the protective action of E2 in the present cytotoxic models, a transfected cell line with ERα and/or ERβ will be important for future research. The transfected cells will also be suitable for illustrating how the balance between ERα and ERβ shifts, and how that change modulates E2 effects in each cytotoxic model.

3). We have shown that an overexpression of the L-type VDCC was the predominant possible mechanism in calcium homeostasis disruption that contributed to

Aβ etiology. However, other mechanisms may also contribute to Aβ induced dysfunction in calcium homeostasis. Another route for Ca^{++} influx in most neurons is through the NMDA receptor, and it has been shown that excessive activation of the NMDA receptor may contribute to neuronal injury [290]. How the NMDA receptor-mediated Ca^{++} influx contributes to cellular calcium homeostasis disturbance is a worthwhile subject for investigation.

4). The current studies have shown that the expression of α_1 subunits of L-type VDCCs are up-regulated in the presence of Aβ. The addition of estrogen and nifedipine significantly reverses the elevated protein expressions, and this phenomenon correlated with the results showing that both E2 and nifedipine restored the cell viability loss induced by Aβ. These observations suggested a possible mechanism for Aβ cytotoxicity: increased L-type VDCC-mediated increased Ca^{++} influx, with the elevated $[Ca^{++}]_i$ leading to cell death. It is also possible that the protective effects of E2 and nifedipine occurred by stabilizing channel protein overexpression. However, the data in the current thesis did not provide a direct link between the elevation of $[Ca^{++}]_i$ through the newly synthesized channels and cell death. Future studies investigating how Aβ-increased channel expression contributes to elevation of $[Ca^{++}]_i$ and the cell death will be important to help revealing the cellular mechanisms of Aβ-pathology and the protective actions of E2 and nifedipine.

5). Oxidative stress, accumulation of ROS, and loss of calcium homeostasis are major causes in neuronal death related to both apoptosis and necrosis. We have demonstrated the calcium homeostasis disruption in the Aβ model, but did not conduct studies on the role of free radical formation in the cytotoxic models. Future experiments could investigate how free radical formation is involved in the neurocytotoxicity and the neuroprotection we have observed. The relationship between loss of calcium homeostasis and ROS accumulation should also be investigated.

Future studies that answer the above questions will be extremely helpful in demonstrating the cellular mechanisms for various brain diseases and injuries, and in evaluating the neuroprotective actions of estrogen.

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Appendix A

We have conducted experiments to illustrate how cellular calcium homeostasis affected neuronal survival related to the neurodegenerative insults, Aβ and MPTP. The results are listed and discussed in Chapter 5.

To compare the other apoptotic models, which are the serum deprivation and high density culture with the degenerative models, we briefly tested the effects of VDCC blockers on cell viability. In serum-free, 1%, or 3%FBS SK-N-SH cell culture none of the channel blockers showed any protective effect against trophic factor withdrawal induced cell death (Fig. A.1 ~ A.3), suggesting that the cytotoxicity in these models was probably not involved in modulation of Ca^{++} influxes through VDCCs. However, in the high density model, nifedipine (5μM) restored cell number by 22.45 ± 6.26% (Fig. A.4), suggesting an involvement of Ca^{++} influxes through L-type channels in an ischemic-like cell death model.

Central to ischemic brain damage research is the concept of cellular energy failure. [526] Inadequate synthesis of ATP has been thought to produce cellular acidosis through stimulation of anaerobic glycolysis, a disruption of ion homeostasis due to the loss of energy-dependent ion transport mechanisms, and a loss of cytoskeletal integrity as a consequence of decreased synthesis of macromolecular assemblies required for maintaining cell structure. Cellular energy failure has been hypothesized to result in Ca^{++} overload though a number of mechanisms, including increased Ca^{++} influx, decreased Ca^{++} efflux, and altered internal Ca^{++} buffering and sequestration. It has been hypothesized that this Ca^{++} overload contributes further to cell death. Ca^{++} channel blockade has been considered as a potential therapeutic strategy for brain ischemia. The suspected beneficial aspects of Ca^{++} channel blockade have been considered in the following. First, the addition of VDCC blockers contributed to improvement of cerebral circulation. [276, 324] Earlier expectations that the Ca^{++} channel antagonists would be effective therapeutic agents for ischemia were derived from the belief that improved blood circulation in brain areas would reverse cellular pathology. The drugs developed were therefore derivatives of the classical Ca^{++} channel antagonists used for cardiovascular diseases, including nimodipine, nicardipine, isradipine, and lemdipine [324]. These blockers were reported to exert protection because of their selectivity for L-type channels in the cerebral artery and neuroprotection from ischemia in animal models. Secondly, the direct blockade of neuronal Ca^{++} channels could be beneficial; however, it has not been studied extensively so far. Thirdly, inhibition of release of certain neurotransmitters was another parameter for mediating neuroprotection. [324] However, in most of the *in vivo* studies from the available literature, the detected neuroprotective effects of Ca^{++} channel blockers were probably through their effects on cardiovascular tissues and improved neuronal function by enhancing blood perfusion. The current study has shown a direct protective effect of nifedipine in a neuronal apoptotic model which mimics an ischemic condition, suggesting that nutrient and energy deprivation induced cell death also involves in disruption in calcium homeostasis, and a blockade of L-type VDCC favors the survival of the neurons against the above insult.

As shown in Chapter 5, MPTP treatment increased the protein expressions of α_{1C} and α_{1D} of L-type VDCCs. However, the increases in these two isotypes were not modified by either E2 or nifedipine. It has been hypothesized that the elevations in α_{1C} and α_{1D} expression might be related to an apoptotic process. A brief experiment was carried out to investigate whether the increased α_{1C} and α_{1D} expression with MPTP which could not be modified with the addition of either E2 or nifedipine was simply due to apoptotic cell death. Western blot with anti-α_{1C} and α_{1D} demonstrated that both high density and serum-free insults could induce a profound increase in α_{1C} (Fig. A.5) and α_{1D} (Fig. A.6) expression. Under basal conditions, a 215Kda band could hardly be detected with anti-α_{1D} in SK-N-SH cells. Treatments with both serum-free and high density significantly increased the expression of the 215Kda molecule of α_{1D} (Fig. A.6, A and C), and increased the expression of the 175Kda molecule (Fig. A.6B) as well. These observations suggested that the observed overexpression of these two isoforms in the above treated culture was probably due to apoptotic cell death.

These results, which revealed that both α_{1C} and α_{1D} expression were elevated when apoptosis occurred, suggested that apoptosis was capable of up-regulating protein expression non-specifically. Apoptosis is a well programmed process involving regulation of various genes and protein synthesis. The overexpression of the α_{1C} and α_{1D} subunits of the L-channel correlated with the occurrence of apoptosis, suggesting some non-specific elevated protein expression related to the programmed neuronal death process.

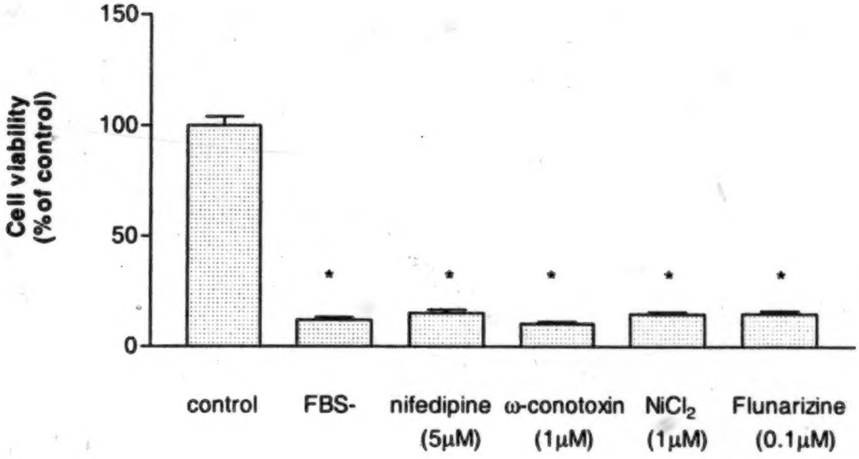

Fig. A.1. The effects of VDCC blockers on serum free-media-treated SK-N-SH cells.

SK-N-SH cells were treated in serum free-media. Cell viability was assayed after 96hr.

*, $p<0.05$, compared with the control group. (n=5)

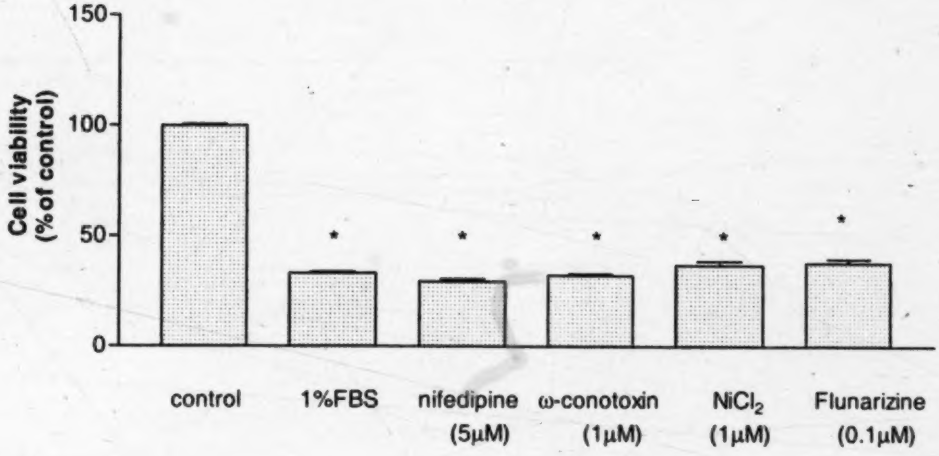

Fig. A.2. The effects of VDCC blockers on 1% FBS media-treated SK-N-SH cells.

SK-N-SH cells were treated in 1%FBS media. Cell viability was assayed after 96hr.

*, $p<0.05$, compared with the control group. (n=5)

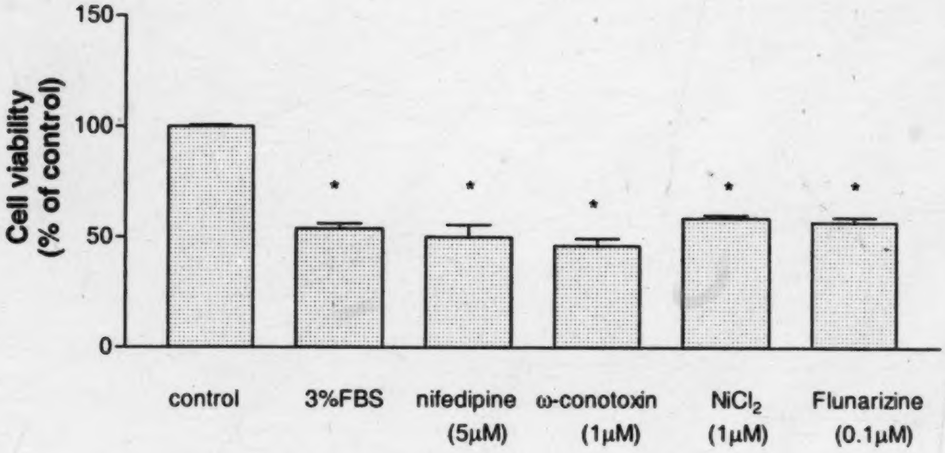

Fig. A.3. The effects of VDCC blockers on 3% FBS media-treated SK-N-SH cells.

SK-N-SH cells were treated in 3%FBS media. Cell viability was assayed after 96hr.

*, $p<0.05$, compared with the control group. (n=4)

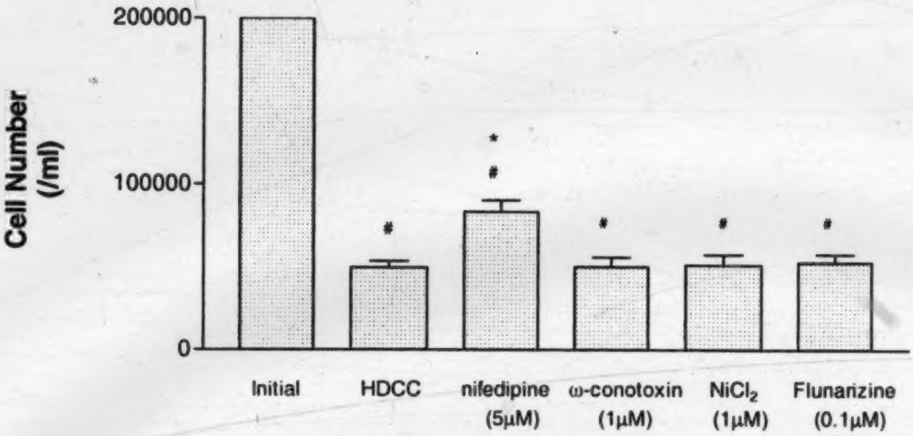

Fig. A.4. The effects of VDCC blockers on high density culture-treated SK-N-SH cells.

SK-N-SH cells were plated at the density of 2×10^5 cells/ml when initiating experiments. Cell viability was assayed after 96hr.

#, $p<0.05$, compared with the initial group;
*, $p<0.05$, compared with the high density culture group. (n=4)

THE EXPRESSION OF α_{1C} IN HIGH DENSITY AND SERUM-FREE GROUP

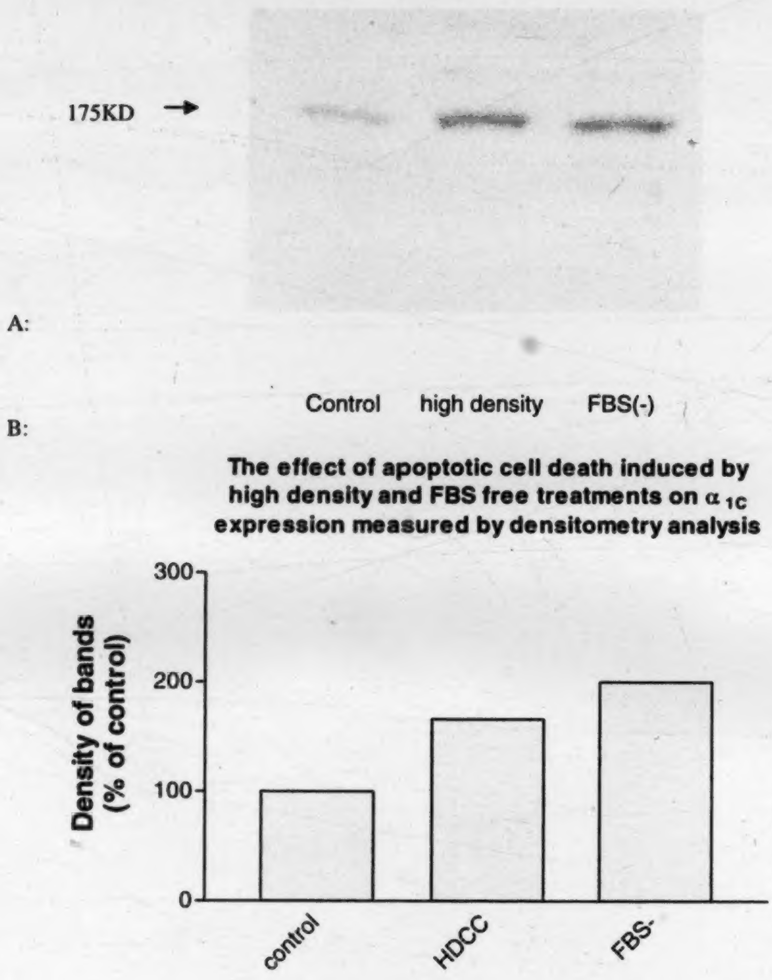

Fig. A.5. The effects of high density and serum-free treatments on the expression of α_{1C} subunit.

A: The effects of high density and serum-free treatments on the expression of α_{1C} subunit of L-type VDCC in SK-N-SH cells by Western blotting with anti- α_{1C} subunit polyclonal antibody.

B: Densitometry analysis of the α_{1C} protein expression in each group.

THE EXPRESSION OF α_{1D} IN HIGH DENSITY AND SERUM-FREE GROUP

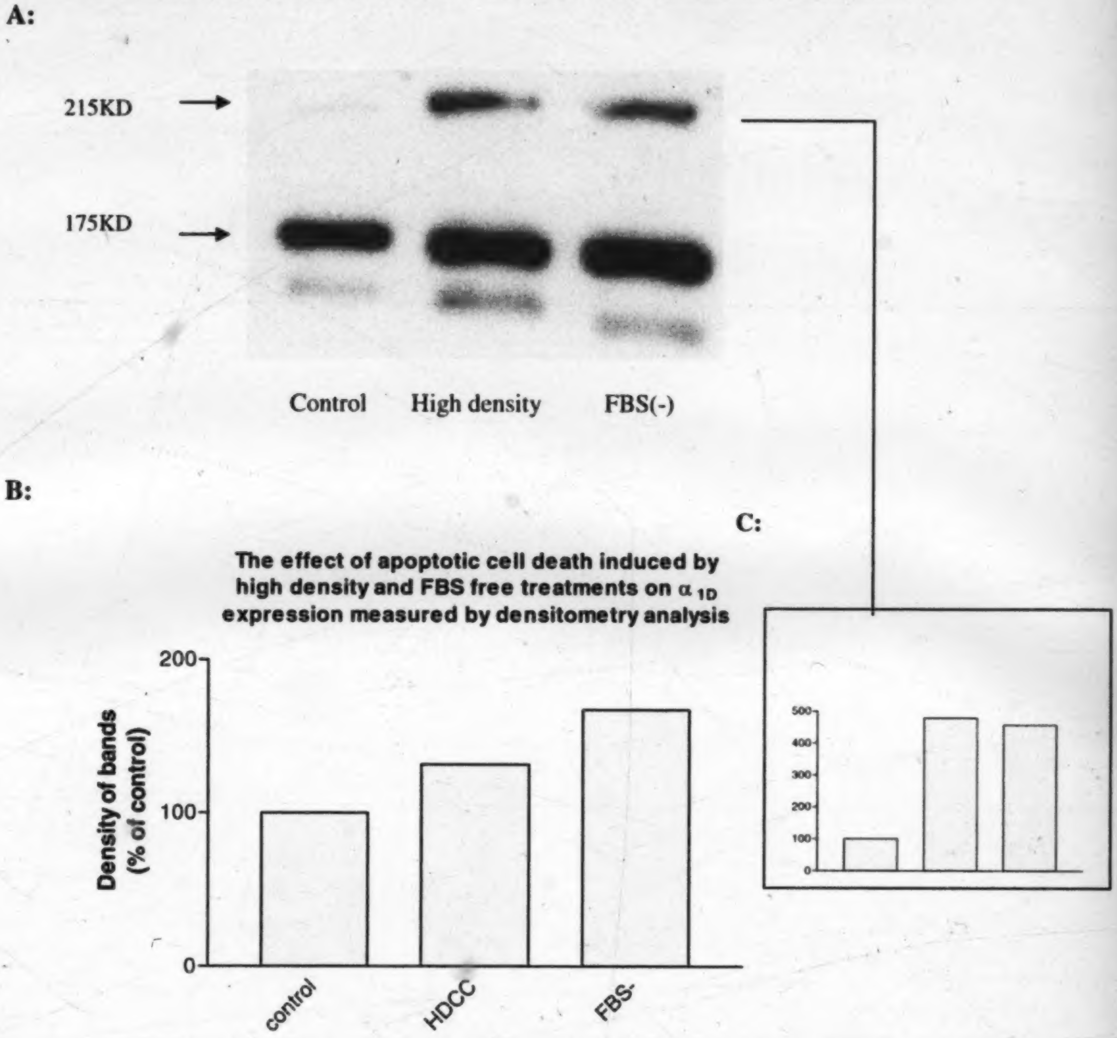

Fig. A.6. The effects of high density and serum-free treatments on the expression of α_{1D} subunit.

A: The effects of high density and serum-free treatments on the expression of α_{1D} subunit of L-type VDCC in SK-N-SH cells by Western blotting with anti- α_{1D} subunit polyclonal antibody.
B: Densitometry analysis of the 175Kda α_{1D} protein expression in each group.
C: Densitometry analysis of the 215Kda α_{1D} protein expression in each group.

Appendix B

As discussed in Chapter 5, SK-N-SH cells express low levels of VDCC. Experiments with patch clamp and fura-2 did not record any L-type VDCC activity. Western blotting analysis with anti-α_1, anti-α_{1C} and anti-α_{1D} detected the expressions of the α_1 DHP binding complex and the targeted two neuronal isotypes of the $\alpha_1$subunits of L-type VDCCs. To confirm that the detected bands represent the relevant α_1 subunit, GH3 cells which express abundant L-type VDCCs and responded to DHP, DHP antagonists, and KCl induced depolarization, were analyzed with Western blotting under the same conditions as that of SK-N-SH cells. The same amounts of the protein preparations from GH3 and SK-N-SH cells were loaded, and the blots were compared. GH3 cells express higher α_1 DHP binding complex (Fig. A.7), and α_{1C} (Fig. A.8) than those of the SK-N-SH cells. This correlates with what has been observed with patch clamp and fura-2 studies. However, GH3 cells have a lower level of α_{1D} protein expression than that of the SK-N-SH cells (Fig. A.9). Since no tracings of the L-type channels and no depolarization-induced Ca^{++} influx have been recorded in the SK-N-SH cells, whether this α_{1D} expression in this line represents a non-functional channel needs to be further illustrated.

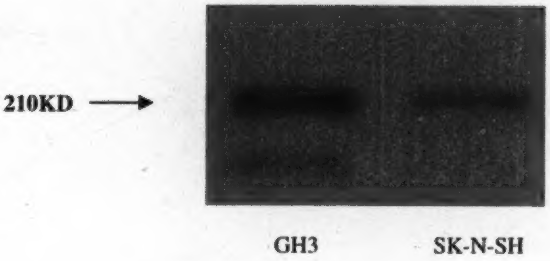

Fig. A.7. The comparison of the expression of the α_1 subunit in GH3 and SK-N-SH cells .

Same amounts of protein preparations (100μg) were loaded to the gel, and the blot was compared.

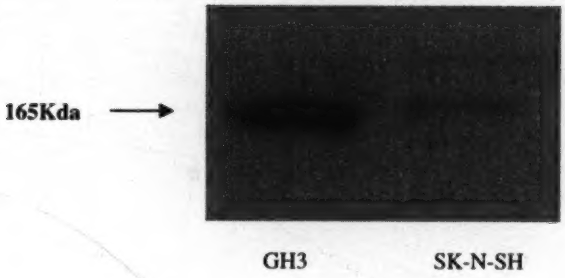

Fig. A.8. The comparison of the expression of the α_{1C} subunit in GH3 and SK-N-SH cells .

Same amounts of protein preparations (100µg) were loaded to the gel, and the blot was compared.

175Kda
GH3
SK-N-SH

Fig. A.9. The comparison of the expression of the α_{1D} subunit in GH3 and SK-N-SH cells .

Same amounts of protein preparations (100μg) were loaded to the gel, and the blot was compared.